IPS A1

CONTROL OF GENE EXPRESSION

FIELD OF THE INVENTION

The present invention relates generally to a method of modifying gene expression and to synthetic genes for modifying endogenous gene expression in a cell, tissue or organ of a transgenic organism, in particular a transgenic animal or plant. More particularly, the present invention utilises recombinant DNA technology to post-transcriptionally modify or modulate the expression of a target gene in a cell, tissue, organ or whole organism, thereby producing novel phenotypes. Novel synthetic genes and genetic constructs which are capable of repressing delaying or otherwise reducing the expression of an endogenous gene or a target gene in an organism when introduced thereto are also provided.

GENERAL

Bibliographic details of the publications referred to in this specification are collected at the end of the description.

As used herein the term "derived from" shall be taken to indicate that a specified integer may be obtained from a particular specified source or species, albeit not necessarily directly from that specified source or species.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated step or element or integer or group of steps or elements or integers but not the exclusion of any other step or element or integer or group of elements or integers.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The

invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

Sequence identity numbers (SEQ ID NOS.) containing nucleotide and amino acid sequence information included in this specification are collected after the Abstract and have been prepared using the programme PatentIn Version 2.0. Each nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc). The length, type of sequence (DNA, protein (PRT), etc) and source organism for each nucleotide or amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification are defined by the information provided in numeric indicator field <400> followed by the sequence identifier (eg. <400>1, <400>2, etc).

The designation of nucleotide residues referred to herein are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents thymine, Y represents a pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K represents Guanine or Thymine, S represents Guanine or Cytosine, W represents Adenine or Thymine, H represents a nucleotide other than Guanine, B represents a nucleotide other than Adenine, V represents a nucleotide other than Thymine, D represents a nucleotide other than Cytosine and N represents any nucleotide residue.

The designation of amino acid residues referred to herein, as recommended by the IUPAC-IUB Biochemical Nomenclature Commission, are listed in Table 1.

TABLE 1

Amino Acid	Three-letter code	One-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Aspartate/Asparagine	Baa	B
Glutamate/Glutamine	Zaa	Z
Any amino acid	Xaa	X

BACKGROUND TO THE INVENTION

Controlling metabolic pathways in eukaryotic organisms is desirable for the purposes of producing novel traits therein or introducing novel traits into a particular cell, tissue or organ of said organism. Whilst recombinant DNA technology has provided significant progress in an understanding of the mechanisms regulating eukaryotic gene expression, much less progress has been made in the actual manipulation of gene expression to produce novel traits. Moreover, there are only limited means by which human intervention may lead to a modulation of the level of eukaryotic gene expression.

One approach to repressing, delaying or otherwise reducing gene expression utilise a mRNA molecule which is transcribed from the complementary strand of a nuclear gene to that which is normally transcribed and capable of being translated into a polypeptide. Although the precise mechanism involved in this approach is not established, it has been postulated that a double-stranded mRNA may form by base pairing between the complementary nucleotide sequences, to produce a complex which is translated at low efficiency and/or degraded by intracellular ribonuclease enzymes prior to being translated.

Alternatively, the expression of an endogenous gene in a cell, tissue or organ may be suppressed when one or more copies of said gene, or one or more copies of a substantially similar gene are introduced into the cell. Whilst the mechanism involved in this phenomenon has not been established and appears to ~~be~~ involve mechanistically heterogeneous processes. For example, this approach has been postulated to involve transcriptional repression, in which case somatically-heritable repressed states of chromatin are formed or alternatively, a post-transcriptional silencing wherein transcription initiation occurs normally but the RNA products of the co-suppressed genes are subsequently eliminated.

The efficiency of both of these approaches in targeting the expression of specific

genes is very low and highly variable results are usually obtained. Inconsistent results are obtained using different regions of genes, for example 5′- untranslated regions, 3′-untranslated regions, coding regions or intron sequences to target gene expression. Accordingly, there currently exists no consensus as to the nature of genetic sequences which provide the most efficient means for repressing, delaying or otherwise reducing gene expression using existing technologies. Moreover, such a high degree of variation exists between generations such that it is not possible to predict the level of repression of a specific gene in the progeny of an organism in which gene expression was markedly modified.

Recently, Dorer and Henikoff (1994) demonstrated the silencing of tandemly repeated gene copies in the *Drosophila* genome and the transcriptional repression of dispersed *Drosophila Adh* genes by *Polycomb* genes (i.e. the *Pc-G* system; Pal-Bhadra *et al*, 1997). However, such silencing of tandemly repeated gene copies is of little utility in an attempt to manipulate gene expression in an animal cell by recombinant means, wherein the sequences capable of targeting the expression of a particular gene are introduced at dispersed locations in the genome, absent the combination of this approach with gene-targeting technology. Whilst theoretically possible, such combinations would be expected to work at only low-efficiency, based upon the low efficiency of gene-targeting approaches used in isolation and further, would require complicated vector systems. Additionally, the utilisation of transcriptional repression, such as the *Drosophila Pc-G* system, would appear to require some knowledge of the regulatory mechanisms capable of modulating the expression of any specific target gene and, as a consequence, would be difficult to implement in practice as a general technology for repressing, delaying or reducing gene expression in animal cells.

The poor understanding of the mechanisms involved in these phenomena has meant that there have been few improvements in technologies for modulating the level of gene expression , in particular technologies for delaying, repressing or otherwise reducing the expression of specific genes using recombinant DNA technology.

Furthermore, as a consequence of the unpredictability of these approaches, there is currently no commercially-viable means for modulating the level of expression of a specific gene in a eukaryotic or prokaryotic organism.

Thus, there exists a need for improved methods of modulating gene expression, in particular repressing, delaying or otherwise reducing gene expression in animal cells for the purpose of introducing novel phenotypic traits thereto. In particular, these methods should provide general means for phenotypic modification, without the necessity for performing concomitant gene-targeting approaches.

SUMMARY OF THE INVENTION

The invention is based in part on the surprising discovery by the inventors that cells which exhibit one or more desired traits can be produced and selected from transformed cells comprising a nucleic acid molecule operably linked to a promoter, wherein the transcription product of the nucleic acid molecule comprises a nucleotide sequence which is substantially identical to the nucleotide sequence of a transcript of an endogenous or non-endogenous target gene, the expression of which is intended to be modulated. The transformed cells are regenerated into whole tissues, organs or organisms capable of exhibiting novel traits, in particular virus resistance and modified expression of endogenous genes.

Accordingly, one aspect of the present invention provides a method of modulating the expression of a target gene in an animal cell, tissue or organ, said method at least comprising the step of introducing to said cell, tissue or organ one or more dispersed nucleic acid molecules or foreign nucleic acid molecules comprising multiple copies of a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or complementary thereto for a time and under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

In a particularly preferred embodiment, the dispersed nucleic acid molecules or foreign nucleic acid molecules comprises a nucleotide sequence which encodes multiple copies of an mRNA molecule which is substantially identical to the nucleotide sequence of the mRNA product of the target gene. More preferably, the multiple copies of the target molecule are tandem direct repeat sequences.

In a more particularly preferred embodiment, the dispersed nucleic acid molecule or foreign nucleic acid molecule is in an expressible form such that it is at least capable of being transcribed to produce mRNA.

The target gene may be a gene which is endogenous to the animal cell or alternatively, a foreign gene such as a viral or foreign genetic sequence, amongst others. Preferably, the target gene is a viral genetic sequence.

The invention is particularly useful in the modulation of eukaryotic gene expression, in particular the modulation of human or animal gene expression and even more particularly in the modulation of expression of genes derived from vertebrate and invertebrate animals, such as insects, aquatic animals (eg. fish, shellfish, molluscs, crustaceans such as crabs, lobsters and prawns, avian animals and mammals, amongst others).

A variety of traits are selectable with appropriate procedures and sufficient numbers of transformed cells. Such traits include, but are not limited to, visible traits, disease-resistance traits, and pathogen-resistance traits. The modulatory effect is applicable to a variety of genes expressed in plants and animals including, for example, endogenous genes responsible for cellular metabolism or cellular transformation, including oncogenes, transcription factors and other genes which encode polypeptides involved in cellular metabolism.

For example, an alteration in the pigment production in mice can be engineered by

targeting the expression of the tyrosinase gene therein. This provides a novel phenotype of albinism in black mice. By targeting genes required for virus replication in a plant cell or an animal cell, a genetic construct which comprises multiple copies of nucleotide sequence encoding a viral replicase, polymerase, coat protein or uncoating gene, or protease protein, may be introduced into a cell where it is expressed, to confer immunity against the virus upon the cell.

In performance of the present invention, the dispersed nucleic acid molecule or foreign nucleic acid molecule will generally comprise a nucleotide sequence having greater than about 85% identity to the target gene sequence, however, a higher homology might produce a more effective modulation of expression of the target gene sequence. Substantially greater homology, or more than about 90% is preferred, and even more preferably about 95% to absolute identity is desirable.

The introduced dispersed nucleic acid molecule or foreign nucleic acid molecule sequence, needing less than absolute homology, also need not be full length, relative to either the primary transcription product or fully processed mRNA of the target gene. A higher homology in a shorter than full length sequence compensates for a longer less homologous sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments will be equally effective. Normally, a sequence of greater than 20-100 nucleotides should be used, though a sequence of greater than about 200-300 nucleotides would be preferred, and a sequence of greater than 500-1000 nucleotides would be especially preferred depending on the size of the target gene.

A second aspect of the present invention provides a synthetic gene which is capable of modifying target gene expression in a cell, tissue or organ of a prokaryotic or eukaryotic organism which is transfected or transformed therewith, wherein said synthetic gene at least comprises a dispersed nucleic acid molecular foreign nucleic acid molecule comprising multiple copies of a nucleotide sequence which is

substantially identical to the nucleotide sequence of said target gene or a derivative thereof or a complementary sequence thereto placed operably under the control of a promoter sequence which is operable in said cell, tissue or organ.

A third aspect of the invention provides a synthetic gene which is capable of modifying the expression of a target gene in a cell, tissue or organ of a prokaryotic or eukaryotic organism which is transfected or transformed therewith, wherein said synthetic gene at least comprises multiple structural gene sequences, wherein each of said structural gene sequences comprises a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a derivative thereof or a complementary sequence thereto and wherein said multiple structural gene sequences are placed operably under the control of a single promoter sequence which is operable in said cell, tissue or organ.

A fourth aspect of the present invention provides a synthetic gene which is capable of modifying the expression of a target gene in a cell, tissue or organ of a prokaryote or eukaryote which is transfected or transformed therewith wherein said synthetic gene at least comprises multiple structural gene sequences wherein each of said structural gene sequences is placed operably under the control of a promoter sequence which is operable in said cell, tissue or organ and wherein each of said structural gene sequences comprises a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a derivative thereof or a complementary sequence thereto.

A fifth aspect of the present invention provides a genetic construct which is capable of modifying the expression of an endogenous gene or target gene in a transformed or transfected cell, tissue or organ wherein said genetic construct at least comprises the synthetic gene of the invention and one or more origins of replication and/or selectable marker gene sequences.

In order to observe many novel traits in multicellular organisms such as plants and animals, in particular those which are tissue-specific or organ-specific or developmentally-regulated, regeneration of a transformed cell carrying the synthetic genes and genetic constructs described herein into a whole organism will be required. Those skilled in the art will be aware that this means growing a whole organism from a transformed plant cell or animal cell, a group of such cells, a tissue or organ. Standard methods for the regeneration of certain plants and animals from isolated cells and tissues are known to those skilled in the art.

Accordingly, a sixth aspect of the invention provides a cell, tissue, organ or organism comprising the synthetic genes and genetic constructs described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagrammatic representation of the plasmid pEGFP-N1 MCS.

Figure 2 is a diagrammatic representation of the plasmid pCMV.cass.

Figure 3 is a diagrammatic representation of the plasmid pCMV.SV40L.cass.

Figure 4 is a diagrammatic representation of the plasmid pCMV.SV40LR.cass.

Figure 5 is a diagrammatic representation of the plasmid pCR.Bgl-GFP-Bam.

Figure 6 is a diagrammatic representation of the plasmid pBSII(SK+).EGFP.

Figure 7 is a diagrammatic representation of the plasmid pCMV.EGFP.

Figure 8 is a diagrammatic representation of the plasmid pCR.SV40L.

Figure 9 is a diagrammatic representation of the plasmid pCR.BEV.1.

Figure 10 is a diagrammatic representation of the plasmid pCR.BEV.2.

Figure 11 is a diagrammatic representation of the plasmid pCR.BEV.3.

Figure 12 is a diagrammatic representation of the plasmid pCMV.EGFP.BEV2.

Figure 13 is a diagrammatic representation of the plasmid pCMV.BEV.2.

Figure 14 is a diagrammatic representation of the plasmid pCMV.BEV.3.

Figure 15 is a diagrammatic representation of the plasmid pCMV.VEB.

Figure 16 is a diagrammatic representation of the plasmid pCMV.BEV.GFP.

Figure 17 is a diagrammatic representation of the plasmid pCMV.BEV.SV40L-0.

Figure 18 is a diagrammatic representation of the plasmid pCMV.0.SV40L.BEV.

Figure 19 is a diagrammatic representation of the plasmid pCMV.0.SV40L.VEB.

Figure 20 is a diagrammatic representation of the plasmid pCMV.BEVx2.

Figure 21 is a diagrammatic representation of the plasmid pCMV.BEVx3.

Figure 22 is a diagrammatic representation of the plasmid pCMV.BEVx4.

Figure 23 is a diagrammatic representation of the plasmid pCMV.BEV.SV40L.BEV.

Figure 24 is a diagrammatic representation of the plasmid pCMV.BEV.SV40L.VEB.

Figure 25 is a diagrammatic representation of the plasmid pCMV.BEV.GFP.VEB.

Figure 26 is a diagrammatic representation of the plasmid pCMV.EGFP.BEV2.PFG.

Figure 27 is a diagrammatic representation of the plasmid pCMV.BEV.SV40LR.

Figure 28 is a diagrammatic representation of the plasmid pCDNA3.Galt.

Figure 29 is a diagrammatic representation of the plasmid pCMV.Galt.

Figure 30 is a diagrammatic representation of the plasmid pCMV.EGFP.Galt.

Figure 31 is a diagrammatic representation of the plasmid pCMV.Galt.GFP.

Figure 32 is a diagrammatic representation of the plasmid pCMV.Galt.SV40L.0.

Figure 33 is a diagrammatic representation of the plasmid pCMV.Galt.SV40L.tlaG.

Figure 34 is a diagrammatic representation of the plasmid pCMV.0.SV40L.Galt.

Figure 35 is a diagrammatic representation of the plasmid pCMV.Galtx2.

Figure 36 is a diagrammatic representation of the plasmid pCMV.Galtx4.

Figure 37 is a diagrammatic representation of the plasmid pCMV.Galt.SV40L.Galt.

Figure 38 is a diagrammatic representation of the plasmid pCMV.Galt.SV40L.tlaG.

Figure 39 is a diagrammatic representation of the plasmid pCMV.Galt.GFP.tlaG.

Figure 40 is a diagrammatic representation of the plasmid pCMV.EGFP.Galt.PFG.

Figure 41 is a diagrammatic representation of the plasmid pCMV.Galt.SV40LR.

Figure 42 is a diagrammatic representation of the plasmid pART7.

Figure 43 is a diagrammatic representation of the plasmid pART7.35S.SCBV.cass.

Figure 44 is a diagrammatic representation of the plasmid pBC.PVY.

Figure 45 is a diagrammatic representation of the plasmid pSP72.PVY.

Figure 46 is a diagrammatic representation of the plasmid pClapBC.PVY.

Figure 47 is a diagrammatic representation of the plasmid pBC.PVYx2.

Figure 48 is a diagrammatic representation of the plasmid pSP72.PVYx2.

Figure 49 is a diagrammatic representation of the plasmid pBC.PVYx3.

Figure 50 is a diagrammatic representation of the plasmid pBC.PVYx4.

Figure 51 is a diagrammatic representation of the plasmid pBC.PVY.LNYV.

Figure 52 is a diagrammatic representation of the plasmid pBC.PVY.LNYV.PVY.

Figure 53 is a diagrammatic representation of the plasmid pBC.PVY.LNYV.YVP$_{\Delta}$.

Figure 54 is a diagrammatic representation of the plasmid pBC.PVY.LNYV.YVP.

Figure 55 is a diagrammatic representation of the plasmid pART27.PVY

Figure 56 is a diagrammatic representation of the plasmid pART27.35S.PVY.SCBV.O.

Figure 57 is a diagrammatic representation of the plasmid pART27.35S.O.SCBV.PVY.

Figure 58 is a diagrammatic representation of the plasmid pART27.35S.O.SCBV.YVP.

Figure 59 is a diagrammatic representation of the plasmid pART7.PVYx2.

Figure 60 is a diagrammatic representation of the plasmid pART7.PVYx3.

Figure 61 is a diagrammatic representation of the plasmid pART7.PVYx4.

Figure 62 is a diagrammatic representation of the plasmid pART7.PVY.LNYV.PVY.

Figure 63 is a diagrammatic representation of the plasmid pART7.PVY.LNYV.YVP$_{\Delta}$.

Figure 64 is a diagrammatic representation of the plasmid pART7.PVY.LNYV.YVP.

Figure 65 is a diagrammatic representation of pART7.35S.PVY.SCBV.YVP.

Figure 66 is a diagrammatic representation of pART7.35S.PVYx3.SCBV.YVPx3.

Figure 67 is a diagrammatic representation of pART7.PVYx3.LNYV.YVPx3.

Figure 68 is a diagrammatic representation of the plasmid pART7.PVYMULTI.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of modulating the expression of a target gene in a cell, tissue or organ, said method at least comprising the step of introducing to said cell, tissue or organ one or more dispersed nucleic acid molecules or foreign nucleic acid molecules comprising multiple copies of a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or complementary thereto for a time and under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

By "multiple copies" is meant that two or more copies of the target gene are presented in close physical connection or juxtaposed, in the same or different orientation, on the same nucleic acid molecule, optionally separated by a stuffer fragment or intergenic region to facilitate secondary structure formation between each repeat where this is required. The stuffer fragment may comprise any combination of nucleotide or amino acid residues, carbohydrate molecules or oligosaccharide molecules or carbon atoms or a homologue, analogue or derivative thereof which is capable of being linked covalently to a nucleic acid molecule.

In a preferred embodiment
~~Preferably, embodiment,~~ the stuffer fragment comprises a sequence of nucleotides or a homologue, analogue or derivative thereof.

More preferably, the stuffer fragment comprises a sequence of nucleotides of at least about 10-50 nucleotides in length, even more preferably at least about 50-100 nucleotides in length and still more preferably at least about 100-500 nucleotides in length.

Wherein the dispersed or foreign nucleic acid molecule comprises intron/exon splice

junction sequences, the stuffer fragment may serve as an intron sequence placed between the 3′-splice site of the structural gene nearer the 5′-end of the gene and the 5′- splice site of the next downstream unit thereof. Alternatively, wherein it is desirable for more than two adjacent nucleotide sequence units of the dispersed foreign nucleic acid molecule to be translated, the stuffer fragment placed there between should not include an in-frame translation stop codon, absent intron/exon splice junction sequences at both ends of the stuffer fragment or the addition of a translation start codon at the 5′ end of each unit, as will be obvious to those skilled in the art.

Preferred stuffer fragments are those which encode detectable marker proteins or biologically-active analogues and derivatives thereof, for example luciferase, β-galacturonase, β-galactosidase, chloramphenicol acetyltransferase or green fluorescent protein, amongst others. Additional stuffer fragments are not excluded.

According to this embodiment, the detectable marker or an analogue or derivative thereof serves to indicate the expression of the synthetic gene of the invention in a cell, tissue or organ by virtue of its ability to confer a specific detectable phenotype thereon, preferably a visually-detectable phenotype.

As used herein, the term "modulating" shall be taken to mean that expression of the target gene is reduced in amplitude and/or the timing of gene expression is delayed and/or the developmental or tissue-specific or cell-specific pattern of target gene expression is altered, compared to the expression of said gene in the absence of the inventive method described herein.

Whilst not limiting the scope of the invention described herein, the present invention is directed to a modulation of gene expression which comprises the repression, delay or reduction in amplitude of target gene expression in a specified cell, tissue or organ of a eukaryotic organism, in particular a plant such as a monocotyledonous or dicotyledonous plant, or a human or other animal and even more particularly a

vertebrate and invertebrate animal, such as an insect, aquatic animal (eg. fish, shellfish, mollusc, crustacean such as a crab, lobster or prawn, an avian animal or a mammal, amongst others).

More preferably, target gene expression is completely inactivated by the dispersed nucleic acid molecules or foreign nucleic acid molecules which has been introduced to the cell, tissue or organ.

Whilst not being bound by any theory or mode of action, the reduced or eliminated expression of the target gene which results from the performance of the invention may be attributed to reduced or delayed translation of the mRNA transcription product of the target gene or alternatively, the prevention of translation of said mRNA, as a consequence of sequence-specific degradation of the mRNA transcript of the target gene by an endogenous host cell system.

It is particularly preferred that, for optimum results, sequence-specific degradation of the mRNA transcript of the target gene occurs either prior to the time or stage when the mRNA transcript of the target gene would normally be translated or alternatively, at the same time as the mRNA transcript of the target gene would normally be translated. Accordingly, the selection of an appropriate promoter sequence to regulate expression of the introduced dispersed nucleic acid molecule or foreign nucleic acid molecule is an important consideration to optimum performance of the invention. For this reason, strong constitutive promoters or inducible promoter systems are especially preferred for use in regulating expression of the introduced dispersed nucleic acid molecules or foreign nucleic acid molecules.

The present invention clearly encompasses reduced expression wherein reduced expression of the target gene is effected by lowered transcription, subject to the proviso that a reduction in transcription is not the sole mechanism by which this occurs and said reduction in transcription is at least accompanied by reduced translation of

the steady-state mRNA pool.

The target gene may be a genetic sequence which is endogenous to the animal cell or alternatively, a non-endogenous genetic sequence, such as a genetic sequence which is derived from a virus or other foreign pathogenic organism and is capable of entering a cell and using the cell's machinery following infection.

Wherein the target gene is a non-endogenous genetic sequence to the animal cell, it is desirable that the target gene encodes a function which is essential for replication or reproduction of the viral or other pathogen. In such embodiments, the present invention is particularly useful in the prophylactic and therapeutic treatment of viral infection of an animal cell or for conferring or stimulating resistance against said pathogen.

Preferably, the target gene comprises one or more nucleotide sequences of a viral pathogen of a plant or an animal cell, tissue or organ.

For example, in the case of animals and humans, the viral pathogen may be a retrovirus, for example a lentivirus such as the immunodeficiency viruses, a single-stranded (+) RNA virus such as bovine enterovirus (BEV) or Sinbis alphavirus. Alternatively, the target gene can comprise one or more nucleotide sequences of a viral pathogen of an animal cell, tissue or organ, such as but not limited to a double-stranded DNA virus such as bovine herpes virus or herpes simplex virus I (HSV I), amongst others.

In the case of plants, the virus pathogen is preferably a potyvirus, caulimovirus, badnavirus, geminivirus, reovirus, rhabdovirus, bunyavirus, tospovirus, tenuivirus, tombusvirus, luteovirus, sobemovirus, bromovirus, cucomovirus, ilavirus, alfamovirus, tobamovirus, tobravirus, potexvirus and clostrovirus, such as but not limited to CaMV, SCSV, PVX, PVY, PLRV, and TMV, amongst others.

With particular regard to viral pathogens, those skilled in the art are aware that virus-encoded functions may be complemented *in trans* by polypeptides encoded by the host cell. For example, the replication of the bovine herpes virus genome in the host cell may be facilitated by host cell DNA polymerases which are capable of complementing an inactivated viral DNA polymerase gene.

Accordingly, wherein the target gene is a non-endogenous genetic sequence to the animal cell, a further alternative embodiment of the invention provides for the target gene to encode a viral or foreign polypeptide which is not capable of being complemented by a host cell function, such as a virus-specific genetic sequence. Exemplary target genes according to this embodiment of the invention include, but are not limited to genes which encode virus coat proteins, uncoating proteins and RNA-dependent DNA polymerases and RNA-dependent RNA polymerases, amongst others.

In a particularly preferred embodiment of the present invention, the target gene is the BEV RNA-dependent RNA polymerase gene or a homologue, analogue or derivative thereof or PVY Nia protease-encoding sequences.

The cell in which expression of the target gene is modified may be any cell which is derived from a multicellular plant or animal, including cell and tissue cultures thereof. Preferably, the animal cell is derived from an insect, reptile, amphibian, bird, human or other mammal. Exemplary animal cells include embryonic stem cells, cultured skin fibroblasts, neuronal cells, somatic cells, haematopoietic stem cells, T-cells and immortalised cell lines such as COS, VERO, HeLa, mouse C127, Chinese hamster ovary (CHO), WI-38, baby hamster kidney (BHK) or MDBK cell lines, amongst others. Such cells and cell lines are readily available to those skilled in the art. Accordingly, the tissue or organ in which expression of the target gene is modified may be any tissue or organ comprising such animal cells.

Preferably the plant cell is derived from a monocotyledonous or dicotyledonous plant

species or a cell line derived therefrom.

As used herein, the term "dispersed nucleic acid molecule" shall be taken to refer to a nucleic acid molecule which comprises one or more multiple copies, preferably tandem direct repeats, of a nucleotide sequence which is substantially identical or complementary to the nucleotide sequence of a gene which originates from the cell, tissue or organ into which said nucleic acid molecule is introduced, wherein said nucleic acid molecule is non-endogenous in the sense that it is introduced to the cell, tissue or organ of an animal via recombinant means and will generally be present as extrachromosomal nucleic acid or alternatively, as integrated chromosomal nucleic acid which is genetically-unlinked to said gene. More particularly, the "dispersed nucleic acid molecule" will comprise chromosomal or extrachromosomal nucleic acid which is unlinked to the target gene against which it is directed in a physical map, by virtue of their not being tandemly-linked or alternatively, occupying a different chromosomal position on the same chromosome or being localised on a different chromosome or present in the cell as an episome, plasmid, cosmid or virus particle.

By "foreign nucleic acid molecule" is meant an isolated nucleic acid molecule which has one or more multiple copies, preferably tandem direct repeats, of a nucleotide sequence which originates from the genetic sequence of an organism which is different from the organism to which the foreign nucleic acid molecule is introduced. This definition encompasses a nucleic acid molecule which originates from a different individual of the same lowest taxonomic grouping (i.e. the same population) as the taxonomic grouping to which said nucleic acid molecule is introduced, as well as a nucleic acid molecule which originates from a different individual of a different taxonomic grouping as the taxonomic grouping to which said nucleic acid molecule is introduced, such as a gene derived from a viral pathogen.

Accordingly, a target gene against which a foreign nucleic acid molecule acts in the performance of the invention may be a nucleic acid molecule which has been

introduced from one organism to another organism using transformation or introgression technologies. Exemplary target genes according to this embodiment of the invention include the green fluorescent protein-encoding gene derived from the jellyfish *Aequoria victoria* (Prasher *et al.*, 1992; International Patent Publication No. WO 95/07463), tyrosinase genes and in particular the murine tyrosinase gene (Kwon *et al.*, 1988), the *Escherichia coli lacI* gene which is capable of encoding a polypeptide repressor of the *lacZ* gene, the porcine α-1,3-galactosyltransferase gene (NCBI Accession No. L36535) exemplified herein, and the PVY and BEV structural genes exemplified herein or a homologue, analogue or derivative of said genes or a complementary nucleotide sequence thereto.

The present invention is further useful for simultaneously targeting the expression of several target genes which are co-expressed in a particular cell, for example by using a dispersed nucleic acid molecule or foreign nucleic acid molecule which comprises nucleotide sequences which are substantially identical to each of said co-expressed target genes.

By "substantially identical" is meant that the introduced dispersed or foreign nucleic acid molecule of the invention and the target gene sequence are sufficiently identical at the nucleotide sequence level to permit base-pairing there between under standard intracellular conditions.

Preferably, the nucleotide sequence of each repeat in the dispersed or foreign nucleic acid molecule of the invention and the nucleotide sequence of a part of the target gene sequence are at least about 80-85% identical at the nucleotide sequence level, more preferably at least about 85-90% identical, even more preferably at least about 90-95% identical and still even more preferably at least about 95-99% or 100% identical at the nucleotide sequence level.

Notwithstanding that the present invention is not limited by the precise number of

repeated sequences in the dispersed nucleic acid molecule or the foreign nucleic acid molecule of the invention, it is to be understood that the present invention requires at least two copies of the target gene sequence to be expressed in the cell.

Preferably, the multiple copies of the target gene sequence are presented in the dispersed nucleic acid molecule or the foreign nucleic acid molecule as tandem inverted repeat sequences and/or tandem direct repeat sequences. Such configurations are exemplified by the "test plasmids" described herein that comprise Galt, BEV or PVY gene regions.

Preferably, the dispersed or foreign nucleic acid molecule which is introduced to the cell, tissue or organ comprises RNA or DNA.

Preferably, the dispersed or foreign nucleic acid molecule further comprises a nucleotide sequence or is complementary to a nucleotide sequence which is capable of encoding an amino acid sequence encoded by the target gene. Even more preferably, the nucleic acid molecule includes one or more ATG or AUG translational start codons.

Standard methods may be used to introduce the dispersed nucleic acid molecule or foreign nucleic acid molecule into the cell, tissue or organ for the purposes of modulating the expression of the target gene. For example, the nucleic acid molecule may be introduced as naked DNA or RNA, optionally encapsulated in a liposome, in a virus particle as attenuated virus or associated with a virus coat or a transport protein or inert carrier such as gold or as a recombinant viral vector or bacterial vector or as a genetic construct, amongst others.

Administration means include injection and oral ingestion (e.g. in medicated food material), amongst others.

The subject nucleic acid molecules may also be delivered by a live delivery system such as using a bacterial expression system optimised for their expression in bacteria which can be incorporated into gut flora. Alternatively, a viral expression system can be employed. In this regard, one form of viral expression is the administration of a live vector generally by spray, feed or water where an infecting effective amount of the live vector (e.g. virus or bacterium) is provided to the animal. Another form of viral expression system is a non-replicating virus vector which is capable of infecting a cell but not replicating therein. The non-replicating viral vector provides a means of introducing to the human or animal subject genetic material for transient expression therein. The mode of administering such a vector is the same as a live viral vector.

The carriers, excipients and/or diluents utilised in delivering the subject nucleic acid molecules to a host cell should be acceptable for human or veterinary applications. Such carriers, excipients and/or diluents are well-known to those skilled in the art. Carriers and/or diluents suitable for veterinary use include any and all solvents, dispersion media, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the composition is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

In an alternative embodiment, the invention provides a method of modulating the expression of a target gene in a cell, tissue or organ, said method at least comprising the steps of:

(i) selecting one or more dispersed nucleic acid molecules or foreign nucleic acid molecules which comprise multiple copies of a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or which is complementary thereto; and

(ii) introducing said dispersed nucleic acid molecules or foreign nucleic acid molecules to said cell, tissue or organ for a time and under conditions sufficient

for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

To select appropriate nucleotide sequences for targeting expression of the target gene, several approaches may be employed. In one embodiment, multiple copies of specific regions of characterised genes may be cloned in operable connection with a suitable promoter and assayed for efficacy in reducing target gene expression. Alternatively, shotgun libraries comprising multiple copies of genetic sequences may be produced and assayed for their efficacy in reducing target gene expression. The advantage associated with the latter approach is that it is not necessary to have any prior knowledge of the significance of any particular target gene in specifying an undesirable phenotype in the cell. For example, shotgun libraries comprising virus sub-genomic fragments may be employed and tested directly for their ability to confer virus immunity on the animal host cell, without prior knowledge of the role which any virus genes play in pathogenesis of the host cell.

As used herein, the term "shotgun library" is a set of diverse nucleotide sequences wherein each member of said set is preferably contained within a suitable plasmid, cosmid, bacteriophage or virus vector molecule which is suitable for maintenance and/or replication in a cellular host. The term "shotgun library" includes a representative library, in which the extent of diversity between the nucleotide sequences is numerous such that all sequences in the genome of the organism from which said nucleotide sequences is derived are present in the "set" or alternatively, a limited library in which there is a lesser degree of diversity between said sequences. The term "shotgun library" further encompasses random nucleotide sequences, wherein the nucleotide sequence comprises viral or cellular genome fragments, amongst others obtained for example by shearing or partial digestion of genomic DNA using restriction endonucleases, amongst other approaches. A "shotgun library" further includes cells, virus particles and bacteriophage particles comprising the

individual nucleotide sequences of the diverse set.

Preferred shotgun libraries according to this embodiment of the invention are "representative libraries", comprising a set of tandem repeated nucleotide sequences derived from a viral pathogen of a plant or an animal.

In a particularly preferred embodiment of the invention, the shotgun library comprises cells, virus particles or bacteriophage particles comprising a diverse set of tandem-repeated nucleotide sequences which encode a diverse set of amino acid sequences, wherein the member of said diverse set of nucleotide sequences are placed operably under the control of a promoter sequence which is capable of directing the expression of said tandem-repeated nucleotide sequence in the cell.

Accordingly, the nucleotide sequence of each unit in the tandem-repeated sequence may comprise at least about 1 to 200 nucleotides in length. The use of larger fragments, particularly employing randomly sheared nucleic acid derived from viral, plant or animal genomes, is not excluded.

The introduced nucleic acid molecule is preferably in an expressible form.

By "expressible form" is meant that the subject nucleic acid molecule is presented in an arrangement such that it may be expressed in the cell, tissue, organ or whole organism, at least at the transcriptional level (i.e. it is expressed in the animal cell to yield at least an mRNA product which is optionally translatable or translated to produce a recombinant peptide, oligopeptide or polypeptide molecule).

By way of exemplification, in order to obtain expression of the dispersed nucleic acid molecule or foreign nucleic acid molecule in the cell, tissue or organ of interest, a synthetic gene or a genetic construct comprising said synthetic gene is produced, wherein said synthetic gene comprises a nucleotide sequence as described *supra* in

operable connection with a promoter sequence which is capable of regulating expression therein. Thus, the subject nucleic acid molecule will be operably connected to one or more regulatory elements sufficient for eukaryotic transcription to occur.

Accordingly, a further alternative embodiment of the invention provides a method of modulating the expression of a target gene in an animal cell, tissue or organ, said method at least comprising the steps of:

(i) selecting one or more dispersed nucleic acid molecules or foreign nucleic acid molecules which comprise multiple copies, preferably tandem repeats, of a nucleotide sequence which is substantially identical to the nucleotide sequence of said target gene or a region thereof or which is complementary thereto;

(ii) producing a synthetic gene comprising said dispersed nucleic acid molecules or foreign nucleic acid molecules;

(iii) introducing said synthetic gene to said cell, tissue or organ; and

(iv) expressing said synthetic gene in said cell, tissue or organ for a time and under conditions sufficient for translation of the mRNA product of said target gene to be modified, subject to the proviso that the transcription of said mRNA product is not exclusively repressed or reduced.

Reference herein to a "gene" or "genes" is to be taken in its broadest context and includes:

(i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5′- and 3′- untranslated sequences); and/or

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) and 5′- and 3′- untranslated sequences of the gene; and/or

(iii) a structural region corresponding to the coding regions (i.e. exons) optionally further comprising untranslated sequences and/or a heterologous promoter sequence which consists of transcriptional and/or translational regulatory regions capable of

conferring expression characteristics on said structural region.

The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of a functional product, in particular a sense or antisense mRNA product or a peptide, oligopeptide or polypeptide or a biologically-active protein.

The term "synthetic gene" refers to a non-naturally occurring gene as hereinbefore defined which preferably comprises at least one or more transcriptional and/or translational regulatory sequences operably linked to a structural gene sequence.

The term "structural gene" shall be taken to refer to a nucleotide sequence which is capable of being transmitted to produce mRNA and optionally, encodes a peptide, oligopeptide, polypeptide or biologically active protein molecule. Those skilled in the art will be aware that not all mRNA is capable of being translated into a peptide, oligopeptide, polypeptide or protein, for example if the mRNA lacks a functional translation start signal or alternatively, if the mRNA is antisense mRNA. The present invention clearly encompasses synthetic genes comprising nucleotide sequences which are not capable of encoding peptides, oligopeptides, polypeptides or biologically-active proteins. In particular, the present inventors have found that such synthetic genes may be advantageous in modifying target gene expression in cells, tissues or organs of a prokaryotic or eukaryotic organism.

The term "structural gene region" refers to that part of a synthetic gene which comprises a dispersed nucleic acid molecule or foreign nucleic acid molecule as described herein which is expressed in a cell, tissue or organ under the control of a promoter sequence to which it is operably connected. A structural gene region may comprise one or more dispersed nucleic acid molecules and/or foreign nucleic acid molecules operably under the control of a single promoter sequence or multiple promoter sequences. Accordingly, the structural gene region of a synthetic gene may comprise a nucleotide sequence which is capable of encoding an amino acid

sequence or is complementary thereto. In this regard, a structural gene region which is used in the performance of the instant invention may also comprise a nucleotide sequence which encodes an animo acid sequence yet lacks a functional translation initiation codon and/or a functional translation stop codon and, as a consequence, does not comprise a complete open reading frame. In the present context, the term "structural gene region" also extends to a non-coding nucleotide sequences, such as 5′- upstream or 3′-downstream sequences of a gene which would not normally be translated in a eukaryotic cell which expresses said gene.

Accordingly, in the context of the present invention, a structural gene region may also comprise a fusion between two or more open reading frames of the same or different genes. In such embodiments, the invention may be used to modulate the expression of one gene, by targeting different non-contiguous regions thereof or alternatively, to simultaneously modulate the expression of several different genes, including different genes of a multigene family. In the case of a fusion nucleic acid molecule which is non-endogenous to the animal cell and in particular comprises two or more nucleotide sequences derived from a viral pathogen, the fusion may provide the added advantage of conferring simultaneous immunity or protection against several pathogens, by targeting the expression of genes in said several pathogens. Alternatively or in addition, the fusion may provide more effective immunity against any pathogen by targeting the expression of more than one gene of that pathogen.

Particularly preferred structural gene regions according to this aspect of the invention are those which include at least one translatable open reading frame, more preferably further including a translational start codon located at the 5′-end of said open reading frame, albeit not necessarily at the 5′-terminus of said structural gene region. In this regard, notwithstanding that the structural gene region may comprise at least one translatable open reading frame and/or AUG or ATG translational start codon, the inclusion of such sequences in no way suggests that the present invention requires translation of the introduced nucleic acid molecule to occur in order to modulate the

expression of the target gene. Whilst not being bound by any theory or mode of action, the inclusion of at least one translatable open reading frame and/or translational start codon in the subject nucleic acid molecule may serve to increase stability of the mRNA transcription product thereof, thereby improving the efficiency of the invention.

The optimum number of structural gene sequences which may be involved in the synthetic gene of the present invention will vary considerably, depending upon the length of each of said structural gene sequences, their orientation and degree of identity to each other. For example, those skilled in the art will be aware of the inherent instability of palindromic nucleotide sequences *in vivo* and the difficulties associated with constructing long synthetic genes comprising inverted repeated nucleotide sequences, because of the tendency for such sequences to recombine *in vivo*. Notwithstanding such difficulties, the optimum number of structural gene sequences to be included in the synthetic genes of the present invention may be determined empirically by those skilled in the art, without any undue experimentation and by following standard procedures such as the construction of the synthetic gene of the invention using recombinase-deficient cell lines, reducing the number of repeated sequences to a level which eliminates or minimises recombination events and by keeping the total length of the multiple structural gene sequence to an acceptable limit, preferably no more than 5-10kb, more preferably no more than 2-5kb and even more preferably no more than 0.5-2.0kb in length.

Wherein the structural gene region comprises more than one dispersed nucleic acid molecule or foreign nucleic acid molecule it shall be referred to herein as a "multiple structural gene region" or similar term. The present invention clearly extends to the use of multiple structural gene regions which preferably comprise a direct repeat sequence, inverted repeat sequence or interrupted palindrome sequence of a particular structural gene, dispersed nucleic acid molecule or foreign nucleic acid molecule, or a fragment thereof.

Each dispersed or foreign nucleic acid molecule contained within the multiple structural gene unit of the subject synthetic gene may comprise a nucleotide sequence which is substantially identical to a different target gene in the same organism. Such an arrangement may be of particular utility when the synthetic gene is intended to provide protection against a pathogen in a cell, tissue or organ, in particular a viral pathogen, by modifying the expression of viral target genes. For example, the multiple structural gene may comprise nucleotide sequences (i.e. two or more dispersed or foreign nucleic acid molecules) which are substantially identical to two or more target genes selected from the list comprising DNA polymerase, RNA polymerase, Nia protease, and coat protein or other target gene which is essential for viral infectivity, replication or reproduction. However, it is preferred with this arrangement that the structural gene units are selected such that the target genes to which they are substantially identical are normally expressed at approximately the same time (or later) in an infected cell, tissue or organ as (than) the multiple structural gene of the subject synthetic gene is expressed under control of the promoter sequence. This means that the promoter controlling expression of the multiple structural gene will usually be selected to confer expression in the cell, tissue or organ over the entire life cycle of the virus when the viral target genes are expressed at different stages of infection.

As with the individual sequence units of a dispersed or foreign nucleic acid molecule, the individual units of the multiple structural gene may be spatially connected in any orientation relative to each other, for example head-to-head, head-to-tail or tail-to-tail and all such configurations are within the scope of the invention.

For expression in eukaryotic cells, the synthetic gene generally comprises, in addition to the nucleic acid molecule of the invention, a promoter and optionally other regulatory sequences designed to facilitate expression of the dispersed nucleic acid molecule or foreign nucleic acid molecule.

Reference herein to a "promoter" is to be taken in its broadest context and includes the

transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. A promoter is usually, but not necessarily, positioned upstream or 5′, of a structural gene region, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene.

In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of a nucleic acid molecule in a cell.

Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression of the sense molecule and/or to alter the spatial expression and/or temporal expression of said sense molecule. For example, regulatory elements which confer copper inducibility may be placed adjacent to a heterologous promoter sequence driving expression of a sense molecule, thereby conferring copper inducibility on the expression of said molecule.

Placing a dispersed or foreign nucleic acid molecule under the regulatory control of a promoter sequence means positioning the said molecule such that expression is controlled by the promoter sequence. Promoters are generally positioned 5′ (upstream) to the genes that they control. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous

gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

Examples of promoters suitable for use in the synthetic genes of the present invention include viral, fungal, bacterial, animal and plant derived promoters capable of functioning in plant, animal, insect, fungal, yeast or bacterial cells. The promoter may regulate the expression of the structural gene component constitutively, or differentially with respect to cell, the tissue or organ in which expression occurs or, with respect to the developmental stage at which expression occurs, or in response to external stimuli such as physiological stresses, or pathogens, or metal ions, amongst others.

Preferably, the promoter is capable of regulating expression of a nucleic acid molecule in a eukaryotic cell, tissue or organ, at least during the period of time over which the target gene is expressed therein and more preferably also immediately preceding the commencement of detectable expression of the target gene in said cell, tissue or organ.

Accordingly, strong constitutive promoters are particularly preferred for the purposes of the present invention or promoters which may be induced by virus infection or the commencement of target gene expression.

Plant-operable and animal-operable promoters are particularly preferred for use in the synthetic genes of the present invention. Examples of preferred promoters include the bacteriophage T7 promoter, bacteriophage T3 promoter, SP6 promoter, *lac* operator-promoter, *tac* promoter, SV40 late promoter, SV40 early promoter, RSV-LTR promoter, CMV IE promoter, CaMV 35S promoter, SCSV promoter, SCBV promoter and the like.

In consideration of the preferred requirement for high-level expression which coincides with expression of the target gene or precedes expression of the target gene, it is

highly desirable that the promoter sequence is a constitutive strong promoter such as the CMV-IE promoter or the SV40 early promoter sequence, the SV40 late promoter sequence, the CaMV 35S promoter, or the SCBV promoter, amongst others. Those skilled in the art will readily be aware of additional promoter sequences other than those specifically described.

In the present context, the terms "in operable connection with" or "operably under the control" or similar shall be taken to indicate that expression of the structural gene region or multiple structural gene region is under the control of the promoter sequence with which it is spatially connected; in a cell, tissue, organ or whole organism.

In a preferred embodiment of the invention, a structural gene region (i.e. dispersed nucleic acid molecule or foreign nucleic acid molecule) or multiple structural gene region is placed operably in connection with a promoter orientation relative to the promoter sequence, such that when it is transcribed an mRNA product is synthesized which, if translated, is capable of encoding a polypeptide product of the target gene or a fragment thereof.

However, the present invention is not to be limited to the use of such an arrangement and the invention clearly extends to the use of synthetic genes and genetic constructs wherein the a structural gene region or multiple structural gene region is placed in the "antisense" orientation relative to the promoter sequence, such that at least a part of the mRNA transcription product thereof is complementary to the mRNA encoded by the target gene or a fragment thereof.

Clearly, as the dispersed nucleic acid molecule, foreign nucleic acid molecule or multiple structural gene region comprises tandem direct and/or inverted repeat sequences of the target gene, all combinations of the above-mentioned configurations are encompassed by the invention.

In an alternative embodiment of the invention, the structural gene region or multiple structural gene region is operably connected to both a first promoter sequence and a second promoter sequence, wherein said promoters are located at the distal and proximal ends thereof such that at least one unit of said a structural gene region or multiple structural gene region is placed in the "sense" orientation relative to the first promoter sequence and in the "antisense" orientation relative to the second promoter sequence. According to this embodiment, it is also preferred that the first and second promoters be different, to prevent competition there between for cellular transcription factors which bind thereto. The advantage of this arrangement is that the effects of transcription from the first and second promoters in reducing target gene expression in the cell may be compared to determine the optimum orientation for each nucleotide sequence tested.

The synthetic gene preferably contains additional regulatory elements for efficient transcription, for example a transcription termination sequence.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3′-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3′-end of a primary transcript. Terminators active in plant cells are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants or synthesized *de novo*.

As with promoter sequences, the terminator may be any terminator sequence which is operable in the cells, tissues or organs in which it is intended to be used.

Examples of terminators particularly suitable for use in the synthetic genes of the present invention include the SV40 polyadenylation signal, the HSV TK polyadenylation signal, the CYC1 terminator, ADH terminator, SPA terminator, nopaline synthase (NOS) gene terminator of *Agrobacterium tumefaciens*, the

terminator of the Cauliflower mosaic virus (CaMV) 35S gene, the *zein* gene terminator from *Zea mays,* the Rubisco small subunit gene (SSU) gene terminator sequences, subclover stunt virus (SCSV) gene sequence terminators, any *rho*-independent *E.coli* terminator, or the *lacZ* alpha terminator, amongst others.

In a particularly preferred embodiment, the terminator is the SV40 polyadenylation signal or the HSV TK polyadenylation signal which are operable in animal cells, tissues and organs, octopine synthase (OCS) or nopaline synthase (NOS) terminator active in plant cells, tissues or organs, or the *lacZ* alpha terminator which is active in prokaryotic cells.

Those skilled in the art will be aware of additional terminator sequences which may be suitable for use in performing the invention. Such sequences may readily be used without any undue experimentation.

Means for introducing (i.e. transfecting or transforming) cells with the synthetic genes described herein or a genetic construct comprising same are well-known to those skilled in the art.

In a further alternative embodiment, a genetic construct is used which comprises two or more structural gene regions or multiple structural gene regions wherein each of said structural gene regions is placed operably under the control of its own promoter sequence. As with other embodiments described herein, the orientation of each structural gene region may be varied to maximise its modulatory effect on target gene expression.

According to this embodiment, the promoters controlling expression of the structural gene unit are preferably different promoter sequences, to reduce competition there between for cellular transcription factors and RNA polymerases. Preferred promoters are selected from those referred to *supra*.

Those skilled in the art will know how to modify the arrangement or configuration of the individual structural genes as described *supra* to regulate their expression from separate promoter sequences.

The synthetic genes described *supra* are capable of being modified further, for example by the inclusion of marker nucleotide sequences encoding a detectable marker enzyme or a functional analogue or derivative thereof, to facilitate detection of the synthetic gene in a cell, tissue or organ in which it is expressed. According to this embodiment, the marker nucleotide sequences will be present in a translatable format and expressed, for example as a fusion polypeptide with the translation product(s) of any one or more of the structural genes or alternatively as a non-fusion polypeptide.

Those skilled in the art will be aware of how to produce the synthetic genes described herein and of the requirements for obtaining the expression thereof, when so desired, in a specific cell or cell-type under the conditions desired. In particular, it will be known to those skilled in the art that the genetic manipulations required to perform the present invention may require the propagation of a genetic construct described herein or a derivative thereof in a prokaryotic cell such as an *E. coli* cell or a plant cell or an animal cell.

The synthetic genes of the present invention may be introduced to a suitable cell, tissue or organ without modification as linear DNA in the form of a genetic construct, optionally contained within a suitable carrier, such as a cell, virus particle or liposome, amongst others. To produce a genetic construct, the synthetic gene of the invention is inserted into a suitable vector or episome molecule, such as a bacteriophage vector, viral vector or a plasmid, cosmid or artificial chromosome vector which is capable of being maintained and/or replicated and/or expressed in the host cell, tissue or organ into which it is subsequently introduced.

Accordingly a further aspect of the invention provides a genetic construct which at

least comprises the synthetic gene according to any one or more of the embodiments described herein and one or more origins of replication and/or selectable marker gene sequences.

Genetic constructs are particularly suitable for the transformation of a eukaryotic cell to introduce novel genetic traits thereto, in addition to the provision of resistance characteristics to viral pathogens. Such additional novel traits may be introduced in a separate genetic construct or, alternatively on the same genetic construct which comprises the synthetic genes described herein. Those skilled in the art will recognise the significant advantages, in particular in terms of reduced genetic manipulations and tissue culture requirements and increased cost-effectiveness, of including genetic sequences which encode such additional traits and the synthetic genes described herein in a single genetic construct.

Usually, an origin of replication or a selectable marker gene suitable for use in bacteria is physically-separated from those genetic sequences contained in the genetic construct which are intended to be expressed or transferred to a eukaryotic cell, or integrated into the genome of a eukaryotic cell.

In a particularly preferred embodiment, the origin of replication is functional in a bacterial cell and comprises the pUC or the ColE1 origin or alternatively the origin of replication is operable in a eukaryotic cell, tissue and more preferably comprises the 2 micron (2μm) origin of replication or the SV40 origin of replication.

As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a genetic construct of the invention or a derivative thereof.

Suitable selectable marker genes contemplated herein include the ampicillin-resistance

gene (Amp^r), tetracycline-resistance gene (Tc^r), bacterial kanamycin-resistance gene (Kan^r), is the zeocin resistance gene (Zeocin is a drug of bleomycin family which is trademark of InVitrogen Corporation), the *AURI-C* gene which confers resistance to the antibiotic aureobasidin A, phosphinothricin-resistance gene, neomycin phosphotransferase gene (*npt*II), hygromycin-resistance gene, β-glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene, green fluorescent protein-encoding gene or the luciferase gene, amongst others.

Preferably, the selectable marker gene is the *npt*II gene or Kan^r gene or green fluorescent protein (GFP)-encoding gene.

Those skilled in the art will be aware of other selectable marker genes useful in the performance of the present invention and the subject invention is not limited by the nature of the selectable marker gene.

The present invention extends to all genetic constructs essentially as described herein, which include further genetic sequences intended for the maintenance and/or replication of said genetic construct in prokaryotes or eukaryotes and/or the integration of said genetic construct or a part thereof into the genome of a eukaryotic cell or organism.

As with dispersed or foreign nucleic acid molecules, standard methods described *supra* may be used to introduce synthetic genes and genetic constructs into the cell, tissue or organ for the purposes of modulating the expression of the target gene, for example liposome-mediated transfection or transformation, transformation of cells with attenuated virus particles or bacterial cells, cell mating, transformation or transfection procedures known to those skilled in the art or described by Ausubel *et al.* (1992).

Additional means for introducing recombinant DNA into plant tissue or cells include, but are not limited to, transformation using $CaCl_2$ and variations thereof, in particular

the method described by Hanahan (1983), direct DNA uptake into protoplasts (Krens *et al*, 1982; Paszkowski *et al*, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al*, 1990) microparticle bombardment, electroporation (Fromm *et al.,* 1985), microinjection of DNA (Crossway *et al.*, 1986), microparticle bombardment of tissue explants or cells (Christou *et al*, 1988; Sanford, 1988), vacuum-infiltration of tissue with nucleic acid, or in the case of plants, T-DNA-mediated transfer from *Agrobacterium* to the plant tissue as described essentially by An *et al.*(1985), Herrera-Estrella *et al.* (1983a, 1983b, 1985).

For microparticle bombardment of cells, a microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and Wolf (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the genetic construct may incorporate a plasmid capable of replicating in the cell to be transformed.

Examples of microparticles suitable for use in such systems include 1 to 5 μm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

In a further embodiment of the present invention, the synthetic genes and genetic constructs described herein are adapted for integration into the genome of a cell in which it is expressed. Those skilled in the art will be aware that, in order to achieve integration of a genetic sequence or genetic construct into the genome of a host cell, certain additional genetic sequences may be required. In the case of plants, left and right border sequences from the T-DNA of the *Agrobacterium tumefaciens* Ti plasmid will generally be required.

The present invention further extends to an isolated cell, tissue or organ comprising

the synthetic gene described herein or a genetic construct comprising same. The present invention extends further to regenerated tissues, organs and whole organisms derived from said cells, tissues and organs and to propagules and progeny thereof.

For example, plants may be regenerated from transformed plant cells or tissues or organs on hormone-containing media and the regenerated plants may take a variety of forms, such as chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., a transformed root stock grafted to an untransformed scion in citrus species). Transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plants may be selfed to give homozygous second generation (or T2) transformed plants, and the T2 plants further propagated through classical breeding techniques.

The present invention is further described with reference to the following non-limiting Examples.

EXAMPLE 1

Genetic constructs comprising BEV polymerase gene sequences linked to the CMV promoter sequence and/or the SV40L promoter sequence

1. Commercial Plasmids

Plasmid pBluescript II (SK+)

Plasmid pBluescript II (SK+) is commercially available from Stratagene and comprises the LacZ promoter sequence and *lac*Z-alpha transcription terminator, with a multiple cloning site for the insertion of structural gene sequences therein. The plasmid further comprises the ColE1 and fl origins of replication and ampicillin-resistance gene.

Plasmid pSVL

Plasmid pSVL is commercially-obtainable from Pharmacia and serves as a source of the SV40 late promoter sequence. The nucleotide sequence of pSVL is also publicly available as GenBank Accession Number U13868.

Plasmid pCR2.1

Plasmid pCR2.1 is commercially available from Invitrogen and comprises the LacZ promoter sequence and *lac*Z-α transcription terminator, with a cloning site for the insertion of structural gene sequences there between. Plasmid pCR2.1 is designed to clone nucleic acid fragments by virtue of the A-overhang frequently synthesized by *Taq* polymerase during the polymerase chain reaction. PCR fragments cloned in this fashion are flanked by two EcoRI sites. The plasmid further comprises the ColE1 and f1 origins of replication and kanamycin-resistance and ampicillin-resistance genes.

Plasmid pEGFP-N1 MCS

Plasmid pEGFP-N1 MCS (Figure 1; Clontech) contains the CMV IE promoter operably connected to an open reading frame encoding a red-shifted variant of wild-type green fluorescent protein (GFP; Prasher *et al.*, 1992; Chalfie *et al.*, 1994; Inouye and Tsuji,

1994), which has been optimised for brighter fluorescence. The specific GFP variant encoded by pEGFP-N1 MCS has been disclosed by Cormack *et al.* (1996). Plasmid pEGFP-N1 MCS contains a multiple cloning site comprising *Bgl*II and *Bam*HI sites and many other restriction endonuclease cleavage sites, located between the CMV IE promoter and the GFP open reading frame. Structural genes cloned into the multiple cloning site will be expressed at the transcriptional level if they lack a functional translation start site, however such structural gene sequences will not be expressed at the protein level (i.e. translated). Structural gene sequences inserted into the multiple cloning site which comprise a functional translation start site will be expressed as GFP fusion polypeptides if they are cloned in-frame with the GFP-encoding sequence. The plasmid further comprises an SV40 polyadenylation signal downstream of the GFP open reading frame to direct proper processing of the 3′-end of mRNA transcribed from the CMV-IE promoter sequence. The plasmid further comprises the SV40 origin of replication functional in animal cells; the neomycin-resistance gene comprising SV40 early promoter (SV40 EP in Figure 1) operably connected to the neomycin/kanamycin-resistance gene derived from Tn5 (Kan/neo in Figure 1) and the HSV thymidine kinase polyadenylation signal (HSV TK poly (A) in Figure 1), for selection of transformed cells on kamanycin, neomycin or G418; the pUC19 origin of replication which is functional in bacterial cells (pUC Ori in Figure 1); and the f1 origin of replication for single-stranded DNA production (f1 Ori in Figure 1).

2. Expression cassettes

Plasmid pCMV.cass

Plasmid pCMV.cass (Figure 2) is an expression cassette for driving expression of a structural gene sequence under control of the CMV-IE promoter sequence. Plasmid pCMV.cass was derived from pEGFP-N1 MCS by deletion of the GFP open reading frame as follows: Plasmid pEGFP-N1 MCS was digested with *Pin*AI and *Not* I, blunt-ended using *Pfu*I polymerase and then re-ligated. Structural gene sequences are cloned into pCMV.cass using the multiple cloning site, which is identical to the multiple cloning site of pEGFP-N1 MCS, except it lacks the *Pin*AI site.

Plasmid pCMV.SV40L.cass

Plasmid pCMV.SV40L.cass (Figure 3) comprises the synthetic poly A site and the SV40 late promoter sequence from plasmid pCR.SV40L (Figure 4), sub-cloned as a *Sal* I fragment, into the *Sal* I site of plasmid pCMV.cass (Figure 2), such that the CMV-IE promoter and SV40 late promoter sequences are capable of directing transcription in the same direction. Accordingly, the synthetic poly(A) site at the 5′ end of the SV40 promoter sequence is used as a transcription terminator for structural genes expressed from the CMV IE promoter in this plasmid, which also provides for the insertion of said structural gene via the multiple cloning site present between the SV40 late promoter and the synthetic poly(A) site (Figure 5). The multiple cloning sites are located behind the CMV-IE and SV40 late promoters, including *Bam*HI and *Bgl*II sites.

Plasmid pCMV.SV40LR.cass

Plasmid pCMV.SV40LR.cass (Figure 4) comprises the SV40 late promoter sequence derived from plasmid pCR.SV40L, sub-cloned as a *Sal*I fragment into the *Sal*I site of the plasmid pCMV.cass (Figure 2), such that the CMV-IE or the SV40 late promoter may drive transcription of a structural gene or a multiple structural gene unit, in the sense or antisense orientation, as desired. A multiple cloning site is positioned between the opposing CMV- IE and SV40 late promoter sequences in this plasmid to facilitate the introduction of a structural gene sequence. In order for expression of a structural gene sequence to occur from this plasmid, it must be introduced with its own transcription termination sequence located at the 3′ end, because there are no transcription termination sequences located between the opposing CMV- IE and SV40 late promoter sequences in this plasmid. Preferably, the structural gene sequence or multiple structural gene unit which is to be introduced into pCMV.SV40LR.cass will comprise both a 5′ and a 3′ polyadenylation signal as follows:

(i) where the structural gene sequence or multiple structural gene unit is to be expressed in the sense orientation from the CMV IE promoter sequence and/or in the antisense orientation from the SV40 late promoter, the 5′ polyadenylation signal will be in the antisense orientation and the 3′

polyadenylation signal will be in the sense orientation; and

(ii) where the structural gene sequence or multiple structural gene unit is to be expressed in the antisense orientation from the CMV IE promoter sequence and/or in the sense orientation from the SV40 late promoter, the 5′ polyadenylation signal will be in the sense orientation and the 3′ polyadenylation signal will be in the antisense orientation.

Alternatively or in addition, suitably-oriented terminator sequences may be placed at the 5′-end of the CMV and SV40L promoters, as shown in Figure 4.

Alternatively, plasmid pCMV.SV40LR.cass is further modified to produce a derivative plasmid which comprises two polyadenylation signals located between the CMV IE and SV40 late promoter sequences, in approriate orientations to facilitate expression of any structural gene located therebetween in the sense or antisense orientation from either the CMV IE promoter or the SV40 promoter sequence. The present invention clearly encompasses such derivatives.

Alternatively appropriately oriented terminators could be placed upstream of the CMV and SV40L promoters such that transcriptional termination could occur after readthrough of each of the two promoters in the antisense orientation.

3. Intermediate Constructs

Plasmid pCR.Bgl-GFP-Bam

Plasmid pCR.Bgl-GFP-Bam (Figure 5) comprises an internal region of the GFP open reading frame derived from plasmid pEGFP-N1 MCS (Figure 1) placed operably under the control of the lacZ promoter. To produce this plasmid, a region of the GFP open reading frame was amplified from pEGFP-N1 MCS using the amplification primers Bgl-GFP and GFP-Bam and cloned into plasmid pCR2.1. The internal GFP-encoding region in plasmid pCR.Bgl-GFP-Bam lacks functional translational start and stop codons.

SUB B1

Plasmid pBSII(SK+).EGFP

Plasmid pBSII(SK+).EGFP (Figure 6) comprises the EGFP open reading frame derived from plasmid pEGFP-N1 MCS (Figure 1) placed operably under the control of the *lacZ* promoter. To produce this plasmid, the EGFP encoding region of pEGFP-N1 MCS was excised as a *Not*1/*Xho*1 fragment and cloned into the *Not*1/*Xho*1 cloning sites of plasmid pBluescript II (SK+).

Plasmid pCMV.EGFP

Plasmid pCMV.EGFP (Figure 7) is capable of expressing the EGFP structural gene under the control of the CMV-IE promoter sequence. To produce this plasmid the EGFP sequence from plasmid pBSII(SK+).EGFP was excised as *Bam*HI/*Sac*I fragment and cloned into the *Bgl*II/*Sac*I sites of plasmid pCMV.cass (Figure 2).

Plasmid pCR.SV40L

Plasmid pCR.SV40L (Figure 8) comprises the SV40 late promoter derived from plasmid pSVL (GenBank Accession No. U13868; Pharmacia), cloned into pCR2.1 (Stratagene). To produce this plasmid, the SV40 late promoter was amplified using the primers SV40-1 and SV40-2 which comprise *Sal* I cloning sites to facilitate sub-cloning of the amplified DNA fragment into pCMV.cass. The primer also contains a synthetic poly (A) site at the 5′ end, such that the amplicification product comprises the synthetic poly(A) site at the 5′ end of the SV40 promoter sequence.

Plasmid pCR.BEV.1

The BEV RNA-dependent RNA polymerase coding region was amplified as a 1,385 bp DNA fragment from a full-length cDNA clone encoding same, using primers designated BEV-1 and BEV-2, under standard amplification conditions. The amplified DNA contained a 5′-*Bgl* II restriction enzyme site, derived from the BEV-1 primer sequence and a 3′*Bam*HI restriction enzyme site, derived from the BEV-2 primer sequence. Additionally, as the BEV-1 primer sequence contains a translation start signal 5′-ATG-3′ engineered at positions 15-17, the amplified BEV polymerase

SUB B3 CONT

structural gene comprises the start site in-frame with BEV polymerase-encoding nucleotide sequences, Thus, the amplified BEV polymerase structural gene comprises the ATG start codon immediately upstream (ie. juxtaposed) to the BEV polymerase-encoding sequence. There is no translation stop codon in the amplified DNA. This plasmid is present as Figure 9.

Plasmid pCR.BEV.2

The complete BEV polymerase coding region was amplified from a full-length cDNA clone encoding same, using primers BEV-1 and BEV-3. Primer BEV-3 comprises a *Bam*HI restriction enzyme site at positions 5 to 10 inclusive and the complement of a translation stop signal at positions 11 to 13. As a consequence, an open reading frame comprising a translation start signal and translation stop signal, contained between the *Bgl* II and *Bam*HI restriction sites. The amplified fragment was cloned into pCR2.1 (Stratagene) to produce plasmid pCR2.BEV.2 (Figure 10).

Plasmid pCR.BEV.3

A non-translatable BEV polymerase structural gene was amplified from a full-length BEV polymerase cDNA clone using the amplification primers BEV-3 and BEV-4. Primer BEV-4 comprises a *Bgl*II cloning site at positions 5-10 and sequences downstream of this *Bgl*II site are homologous to nucleotide sequences of the BEV polymerase gene. There is no functional ATG start codon in the amplified DNA product of primers BEV-3 and BEV-4. The BEV polymerase is expressed as part of a polyprotein and, as a consequence, there is no ATG translation start site in this gene. The amplified DNA was cloned into plasmid pCR2.1 (Stratagene) to yield plasmid pCR.BEV.3 (Figure 11).

SUB B4

Plasmid pCMV.EGFP.BEV2

Plasmid pCMV.EGFP.BEV2 (Figure 12) was produced by cloning the BEV polymerase sequence from pCR.BEV.2 as a BglII/BamHI fragment into the BamHI site of pCMV.EGFP.

4. Control Plasmids

Plasmid pCMV.BEV.2

Plasmid pCMV.BEV.2 (Figure 13) is capable of expressing the entire BEV polymerase open reading frame under the control of CMV-IE promoter sequence. To produce pCMV.BEV.2, the BEV polymerase sequence from pCR.BEV.2 was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II/*Bam*HI-digested pCMV.cass (Figure 2).

Plasmid pCMV.BEV.3

Plasmid pCMV.BEV.3 (Figure 14) expresses a non-translatable BEV polymerase structural gene in the sense orientation under the control of the CMV-IE promoter sequence. To produce pCMV.BEVnt, the BEV polymerase sequence from pCR.BEV.3 was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II/*Bam*HI-digested pCMV.cass (Figure 2).

Plasmid pCMV.VEB

Plasmid pCMV.VEB (Figure 15) expresses an antisense BEV polymerase mRNA under the control of the CMV-IE promoter sequence. To produce plasmid pCMV.VEB, the BEV polymerase sequence from pCR.BEV.2 was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II/*Bam*HI-digested pCMV.cass (Figure 2).

Plasmid pCMV.BEV.GFP

Plasmid pCMV.BEV.GFP (Figure 16) was constructed by cloning the GFP fragment from pCR.Bgl-GFP-Bam as a BglII/BamHI fragment into BamHI-digested pCMV.BEV.2. This plasmid serves as a control in some experiments and also as an intermediate construct.

Plasmid pCMV.BEV.SV40-L

Plasmid pCMV.BEV.SV40-L (Figure 17) comprises a translatable BEV polymerase

structural gene derived from plasmid pCR.BEV.2 inserted in the sense orientation between the CMV-IE promoter and the SV40 late promoter sequences of plasmid pCMV.SV40L.cass. To produce plasmid pCMV.BEV.SV40L-O, the BEV polymerase structural gene was sub-cloned as a *Bgl*II-to-*Bam*HI fragment into *Bgl*II-digested pCMV.SV40L.cass DNA.

Plasmid pCMV.O.SV40L.BEV

Plasmid pCMV.O.SV40L.BEV (Figure 18) comprises a translatable BEV polymerase structural gene derived from plasmid pCR.BEV.2 cloned downstream of tandem CMV-IE promoter and SV40 late promoter sequences present in plasmid pCMV.SV40L.cass. To produce plasmid pCMV.O.SV40L.BEV, the BEV polymerase structural gene was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.SV40L.cass DNA.

Plasmid pCMV.O.SV40L.VEB

Plasmid pCMV.O.SV40L.VEB (Figure 19) comprises an antisense BEV polymerase structural gene derived from plasmid pCR.BEV.2 cloned downstream of tandem CMV-IE promoter and SV40 late promoter sequences present in plasmid pCMV.SV40L.cass. To produce plasmid pCMV.O.SV40L.VEB, the BEV polymerase structural gene was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.SV40L.cass DNA.

5. Test Plasmids

Plasmid pCMV.BEVx2

Plasmid pCMV.BEVx2 (Figure 20) comprises a direct repeat of a complete BEV polymerase open reading frame under the control of the CMV-IE promoter sequence. In eukaryotic cells at least, the open reading frame located nearer the CMV-IE promoter is translatable. To produce pCMV.BEVx2, the BEV polymerase structural gene from plasmid pCR.BEV.2 was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.BEV.2, immediately downstream of the

BEV polymerase structural gene already present therein.

Plasmid pCMV.BEVx3

Plasmid pCMV.BEVx3 (Figure 21) comprises a direct repeat of three complete BEV polymerase open reading frames under the control of the CMV-1E promoter. To produce pCMV.BEVx3, the BEV polymerase fragment from pCR.BEV.2 was cloned in the sense orientation as a BglII/BamHI fragment into the BamHI site of pCMV.BEVx2, immediately downstream of the BEV polymerase sequences already present therein.

Plasmid pCMV.BEVx4

Plasmid pCMV.BEVx4 (Figure 22) comprises a direct repeat of four complete BEV polymerase open reading frames under the control of the CMV-1E promoter. To produce pCMV.BEVx4, the BEV polymerase fragment from pCR.BEV.2 was cloned in the sense orientation as a BglII/BamHI fragment into the BamHI site of pCMV.BEVx3, immediately downstream of the BEV polymerase sequences already present therein.

Plasmid pCMV.BEV.SV40L.BEV

Plasmid pCMV.BEV.SV40L.BEV(Figure 23) comprises a multiple structural gene unit comprising two BEV polymerase structural genes placed operably and separately under control of the CMV-IE promoter and SV40 late promoter sequences. To produce plasmid pCMV.BEV.SV40L.BEV, the translatable BEV polymerase structural gene present in pCR.BEV.2 was sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment behind the SV40 late promoter sequence present in *Bam*HI-digested pCMV.BEV.SV40L-O.

Plasmid pCMV.BEV.SV40L.VEB

Plasmid pCMV.BEV.SV40L.VEB (Figure 24) comprises a multiple structural gene unit comprising two BEV polymerase structural genes placed operably and separately

under control of the CMV-IE promoter and SV40 late promoter sequences. To produce plasmid pCMV.BEV.SV40L.VEB, the translatable BEV polymerase structural gene present in pCR.BEV.2 was sub-cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment behind the SV40 late promoter sequence present in *Bam*HI-digested pCMV.BEV.SV40L-O. In this plasmid, the BEV polymerase structural gene is expressed in the sense orientation under control of the CMV-IE promoter to produce a translatable mRNA, whilst the BEV polymerase structural gene is also expressed under control of the SV40 promoter to produce an antisense mRNA species.

Plasmid pCMV.BEV.GFP.VEB

Plasmid pCMV.BEV.GFP.VEB (Figure 25) comprises a BEV structural gene inverted repeat or palindrome, interrupted by the insertion of a GFP open reading frame (stuffer fragment) between each BEV structural gene sequence in the inverted repeat. To produce plasmid pCMV.BEV.GFP.VEB, the GFP stuffer fragment from pCR.Bgl-GFP-Bam was first sub-cloned in the sense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.BEV.2 to produce an intermediate plasmid pCMV.BEV.GFP wherein the BEV polymerase-encoding and GFP-encoding sequences are contained within the same 5′-*Bgl*II-to-*Bam*HI-3′ fragment. The BEV polymerase structural gene from pCMV.BEV.2 was then cloned in the antisense orientation as a *Bgl*II-to-*Bam*HI fragment into *Bam*HI-digested pCMV.BEV.GFP. The BEV polymerase structural gene nearer the CMV-IE promoter sequence in plasmid pCMV.BEV.GFP.VEB is capable of being translated, at least in eukaryotic cells.

Plasmid pCMV.EGFP.BEV2.PFG

Plasmid pCMV.EGFP.BEV2.PFG (Figure 26) comprise a GFP palindrome, interrupted by the insertion of a BEV polymerase sequence between each GFP structural gene in the inverted repeat. To produce this plasmid the GFP fragment from pCR.Bgl-GFP-Bam was cloned as a BglII/BamHI fragment into the BamHI site of pCMV.EGFP.BEV2 in the antisense orientation relative to the CMV promoter.

Plasmid pCMV.BEV.SV40LR

Plasmid pCMV.BEV.SV40LR (Figure 27) comprises a structural gene comprising the entire BEV polymerase open reading frame placed operably and separately under control of opposing CMV-IE promoter and SV40 late promoter sequences, thereby potentially producing BEV polymerase transcripts at least from both strands of the full-length BEV polymerase structural gene. To produce plasmid pCMV.BEV.SV40LR, the translatable BEV polymerase structural gene present in pCR.BEV.2 was sub-cloned, as a *Bgl*II-to-*Bam*HI fragment, into the unique *Bgl*II site of plasmid pCMV.SV40LR.cass, such that the BEV open reading frame is present in the sense orientation relative to the CMV-IE promoter sequence.

Those skilled in the art will recognise that it is possible to generate a plasmid wherein the BEV polymerase fragment from pCR.BEV.2 is inserted in the antisense orientation, relative to the CMV IE promoter sequence, using this cloning strategy. The present invention further encompasses such a genetic construct.

EXAMPLE 2

Genetic constructs comprising the porcine α-1,3-galactosyltransferase (Galt) structural gene sequence or sequences operably connected to the CMV promoter sequence and/or the SV40L promoter sequence

1. Commercial Plasmids

Plasmid pcDNA3

Plasmid pcDNA3 is commercially available from Invitrogen and comprises the CMV-IE promoter and BGHpA transcription terminator, with multiple cloning sites for the insertion of structural gene sequences there between. The plasmid further comprises the ColE1 and fl origins of replication and neomycin-resistance and ampicillin-resistance genes.

2. Intermediate plasmids

Plasmid pcDNA3.Galt

Plasmid pcDNA3.Galt (BresaGen Limited, South Australia, Australia; Figure 28) is plasmid pcDNA3 (Invitrogen) and comprises the cDNA sequence encoding porcine gene alpha-1,3-galactosyltransferase (Galt) operably under the control of the CMV-IE promoter sequence such that it is capable of being expressed therefrom. To produce plasmid pcDNA3.Galt, the porcine gene alpha-1,3-galactosyltransferase cDNA was cloned as an *Eco*RI fragment into the *Eco*RI cloning site of pcDNA3. The plasmid further comprises the ColE1 and fl origins of replication and the neomycin and ampicillin-resistance genes.

3. Control Plasmids

Plasmid pCMV.Galt

Plasmid pCMV.Galt (Figure 29) is capable of expressing the Galt structural gene under the control of the CMV-IE promoter sequence. To produce plasmid pCMV.Galt, the Galt sequence from plasmid pcDNA3.Galt was excised as an *Eco*RI fragment and cloned in the sense orientation into the *Eco*RI site of plasmid pCMV.cass (Figure 2).

Plasmid pCMV.EGFP.Galt

Plasmid pCMV.EGFP.Galt (Figure 30) is capable of expressing the Galt structural gene as a Galt fusion polypeptide under the control of the CMV-IE promoter sequence. To produce plasmid pCMV.EGFP.Galt, the Galt sequence from pCMV.Galt (Figure 29) was excised as a *Bgl*II/*Bam*HI fragment and cloned into the *Bam*HI site of pCMV.EGFP.

Plasmid pCMV.Galt.GFP

Plasmid pCMV.Galt.GFP (Figure 31) was produced by cloning the Galt cDNA as an EcORI fragment from pCDNA3 into EcoRI-digested pCMV.EGFP in the sense orientation. This plasmid serves as both a control and construct intermediate.

Plasmid pCMV.Galt.SV40L.0

The plasmid pCMV.Galt.SV40L.0 (Figure 32) comprises a Galt structural gene cloned downstream of the CMV promoter present in pCMV.SV40L.cass. To produce the plasmid the Galt cDNA fragment from pCMV.Galt was cloned as a BglII/BamHI into BglII-digested pCMV.SV40L.cass in the sense orientation.

Plasmid pCMV.O.SV40L.tlaG

The plasmid pCMV.O.SV40L.tlaG (Figure 33) comprises a Galt structural gene clones in an antisense orientation downstream of the SV40L promoter present in pCMV.SV40L.cass. To produce this plasmid the Galt cDNA fragment from pCMV.Galt was cloned as a BglII/BamHI into BamHI-digested pCMV.SV40L.cass in the antisense orientation.

Plasmid pCMV.O.SV40L.Galt

The plasmid pCMV.O.SV40L.Galt (Figure 34) comprises a Galt structural gene cloned downstream of the SV40L promoter present in pCMV.SV40L.cass. To produce the plasmid the Galt cDNA fragment from pCMV.Galt was cloned as a BglII/BamHI into BamHI-digested pCMV.SV40L.cass in the sense orientation.

4. Test Plasmids

Plasmid pCMV.Galtx2

Plasmid pCMV.Galtx2 (Figure 35) comprises a direct repeat of a Galt open reading frame under the control of the CMV-IE promoter sequence. In eukaryotes cells at least, the open reading frame located nearer the CMV-IE promoter is translatable. To produce pCMV.Galtx2, the Galt structural gene from pCMV.Galt was excised as a *Bgl*II/*Bam*HI fragment and cloned in the sense orientation into the *Bam*HI cloning site of pCMV.Galt.

Plasmid pCMV.Galtx4

Plasmid pCMV.Galtx4 (Figure 36) comprises a quadruple direct repeat of a Galt open

reading frame under the control of the CMV-IE promoter sequence. In eukaryotes cells at least, the open reading frame located nearer the CMV-IE promoter is translatable. To produce pCMV.Galtx4, the Galtx2 sequence from pCMV.Galtx2 was excised as a *Bgl*II/*Bam*HI fragment and cloned in the sense orientation into the *Bam*HI cloning site of pCMV.Galtx2.

Plasmid pCMV.Galt.SV40L.Galt

The plasmid pCMV.Galt.SV40L.Galt (Figure 37) is designed to express two sense transcripts of Galt, one driven by the CMV promoter, the other by the SV40L promoter. To produce the plasmid a Galt cDNA fragment from pCMV.Galt was cloned as a BglII/BamHI fragment into BglII-digested pCMV.O.SV40.Galt in the sense orientation.

Plasmid pCMV.Galt.SV40L.tlaG

The plasmid pCMV.Galt.SV40.tlaG (Figure 38) is designed to express a sense transcript of Galt driven by the CMV promoter and an antisense transcript driven by the SV40L promoter. To produce the plasmid a Galt cDNA fragment from pCMV.Galt was cloned as a BglII/BamHI fragment into BglII-digested pCMV.O.SV40.talG in the sense orientation.

Plasmid pCMV.Galt.GFP.tlaG

Plasmid pCMV.Galt.GFP.tlaG (Figure 39) comprise a Galt palindrome, interrupted by the insertion of a GFP sequence between each Galt structural gene in the inverted repeat. To produce this plasmid the BglII/BamHI Galt cDNA fragment from pCMV.Galt was cloned into the BamHI site of pCMV.Galt.GFP in the antisense relative to the CMV promoter.

Plasmid pCMV.EGFP.Galt.PFG

The plasmid pCMV.EGFP.Galt.PFG (Figure 40) comprises a GFP palindrome, interrupted by the insertion of a Galt sequence between each GFP structural gene of the inverted repeat, expression of which is driven by the CMV promoter. To produce

this plasmid the Galt sequences from pCMV.Galt were cloned as a BglII/BamHI fragment into BamHI-digested pCMV.EGFP in the sense orientation to produce the intermediate pCMV.EGFP.Galt (not shown); following this further GFP sequences from pCR.Bgl-pCMV.EGFP.Galt in the antisense orientation.

Plasmid pCMV.Galt.SV40LR

The plasmid pCMV.Galt.SV40LR (Figure 41) is designed to express GalT cDNA sequences cloned between the opposing CMV and SV40L promoters in the expression cassette pCMV.SV40LR.cass. To produce this plasmid Galt sequences from pCMV.Galt were cloned as a BglII/BamHI fragment in BglII-digested pCMV.SV40LR.cass in the sense orientation relative to the 35S promoter.

EXAMPLE 3

Genetic constructs comprising PVY Nia sequences operably linked to the35S promoter sequence and/or the SCBV promoter sequence

1: Binary vector

Plasmid pART27

Plasmid pART27 is a binary vector, specifically designed to be compatible with the pART7 expression cassette. It contains bacterial origins of replication for both E. coli and Agrobacterium tumefaciens, a spectinomycin resistance gene for bacterial selection, left and right T-DNA borders for transfer of DNA from Agrobacterium to plant cells and a kanamycin resistance cassette to permit selection of transformed plant cells. The kanamycin resistance cassette is located between the T-DNA borders, pART27 also contains a unique NotI restriction site which permits cloning of constructs prepared in vectors such as pART7 to be cloned between the T-DNA borders. Construction of pART27 is described in Gleave, AP (1992).

When cloning NotI inserts into this vector, two insert orientations can be obtained. In all the following examples the same insert orientation, relative to the direction of the

35S promoter in the described pART7 constructs was chosen; this was done to minimise any experimental artefacts that may arise from comparing different constructs with different insert orientations.

2. Commercial plasmids

Plasmid pBC (KS-)

Plasmid pBC (KS-) is commercially available from Stratagene and comprises the LacZ promoter sequence and lacZ-alpha transcription terminator, with a multiple cloning site for the insertion of structural gene sequences therein. The plasmid further comprises the ColE1 and fl origins of replication and a chloroamphenicol-resistance gene.

Plasmid pSP72

Plasmid pSP72 is commercially available from Promega and contains a multiple cloning site for the insertion of structural gene sequences therein. The plasmid further comprises the ColE1 origin of replication and an ampicillin-resistance gene.

3. Expression cassettes

Plasmid pART7

Plasmid pART7 is an expression cassette designed to drive expression of sequences cloned behind the 35S promoter. It contains a polylinker to assist cloning and a region of the octipine synthase terminator. The 35S expression cassette is flanked by two Not I restriction sites which permits cloning into binary expression vectors, such as pART27 which contains a unique NotI site. Its construction as described in Gleave, AP (1992), a map is shown in Figure 43.

Plasmid pART7.35S.SCBV.cass

Plasmid p35S.CMV.cass was designed to express two separate gene sequences cloned into a single plasmid. To create this plasmid, sequences corresponding to the nos terminator and the SCBV promoter were amplified by PCR then cloned in the polylinker of pART7 between the 35S promoter and OCS.

The resulting plasmid has the following arrangement of elements:

35S promoter - polylinker 1 - NOS terminator - SCBV promoter - polylinker 2 - OCS terminator.

Expression of sequences cloned into polylinker 1 is controlled by the 35S promoter, expression of sequences cloned into polylinker 2 is controlled by the SCBV promoter.

The NOS terminator sequences were amplified from the plasmid pAHC27 (Christensen and Quail, 1996) using the two oligonucleotides;

NOS 5′ (forward primer; SEQ ID ??)
5′-GGATTCCCGGGACGTCGCGAATTTCCCCCGATCGTTC-3′; and

NOS 3′ (reverse primer; SEQ ID ??)
5′-CCATGGCCATATAGGCCCGATCTAGTAACATAG-3′

Nucleotide residues 1 to 17 for NOS 5′ and 1 to 15 for NOS 3′ represent additional nucleotides designed to assist in construct preparation by adding additional restriction sites. For NOS 5′ these are BamHI, SmaI, AatII and the first 4 bases of an NruI site, for NOS 3′ these are NcoI and SfiI sites. The remaining sequences for each oligonucleotide are homologous to the 5′ and 3′ ends respectively of NOS sequences in pAHC 27.

The SCBV promoter sequences were amplified from the plasmid pScBV-20 (Tzafir *et al*, 1998) using the two oligonucleotides:

SCBV 5′: 5′-CCATGGCCTATATGGCCATTCCCCACATTCAAG-3′; and

SCBV 3′: 5′-AACGTTAACTTCTACCCAGTTCCAGAG-3′

Nucleotide residues 1 to 17 of SCBV 5′ encode NcoI and SfiI restriction sites designed to assist in construct preparation, the remaining sequences are homologous to upstream sequences of the SCMV promoter region. Nucleotide residues 1 to 9 of SCBV 3′ encode Psp10461 and HpaI restriction sites designed to assist in construct preparation, the remaining sequences are homologous to the reverse and complement of sequences near the transcription initiation site of SCBV.

Sequences amplified from pScBV-20 using PCR and cloned into pCR2.1 (Invitrogen) to produce pCR.NOS and pCR.SCBV respectively. SmaI I/SfiI cut pCR.NOS and SfiI/HpaI cut pCR.SCBV were ligated into Sma I cut pART7 and a plasmid with a suitable orientation was chosen and designated pART7.35S.SCBV.cass, a map of this construct is shown in Figure 43.

4. Intermediate constructs

Plasmid pBC.PVY

A region of the PVY genome was amplified by PCR using reverse-transcribed RNA isolated from PVY-infected tobacco as a template using standard protocols and cloned into a plasmid pGEM 3 (Stratagene), to create pGEM.PVY. A SalI/HindIII fragment from pGEM.PVY, corresponding to a SalI/HindIII fragment positions 1536-2270 of the PVY strain O sequence (Acc. No D12539, Genbank), was then subcloned into the plasmid pBC (Stratagene Inc.) to create pBC.PVY (Figure 44).

Plasmid pSP72.PVY

Plasmid pSP72.PVY was prepared by inserting an EcoRI/SalI fragment from pBC.PVY into EcoRI/SalI cut pSP72 (Promega). This construct contains additional restriction sites flanking the PVY insert which were used to assist subsequent manipulations. A map of this construct is shown in Figure 45.

Plasmid ClapBC.PVY

Plasmid Cla pBC.PVY was prepared by inserting a ClaI/SalI fragment from pSP72.PVY

into ClaI/Sal I cutpBC (Stratagene). This construct contains additional restriction sites flanking the PVY insert which were used to assist subsequent manipulations. A map of this construct is shown in Figure 46.

Plasmid pBC.PVYx2

Plasmid pBC.PVYx2 contains two direct head-to-tail repeats of the PVY sequences derived from pBC.PVY. The plasmid was generated by cloning an AccI/ClaI PVY fragment from pSP72.PVY into AccI cut pBC.PVY and is shown in Figure 47.

Plasmid pSP72.PVYx2

Plasmid pSP72.PVYx2 contains two direct head-to-tail repeats of the PVY sequences derived from pBC.PVY. The plasmid was generated by cloning an AccI/ClaI PVY fragment from pBc.PVY into AccI cut pSP72.PVY and is shown in Figure 48.

Plasmid pBC.PVYx3

Plasmid pBC.PVYx3 contains three direct head-to-tail repeats of the PVY sequences derived from pBC.PVY. The plasmid was prepared by cloning an AccI/ClaI PVY fragment from pSP72.PVY into AccI cut pBC.PVYx2 and is shown in Figure 49.

Plasmid pBC.PVYx4

Plasmid pBC.PVYx4 contains four direct head-to-tail repeats of the PVY sequences derived from pBC.PVY. The plasmid was prepared by cloning the direct repeat of PVY sequences from pSP72.PVYx2 as an AccI/ClaI fragment into AccI cut pBC.PVYx2 and is shown in Figure 50.

Plasmid pBC.PVY.LNYV

All attempts to create direct palindromes of PVY sequences failed, presumably such sequence arrangements are unstable in commonly used E. coli cloning hosts. Interrupted palindromes however proved stable.

To create interrupted palindromes of PVY sequences a "stuffer" fragment of approximately 360 bp was inserted into Cla pBV.PVY downstream of the PVY sequences. The stuffer fragment was made as follows:

A clone obtained initially from a cDNA library prepared from lettuce necrotic yellows virus (LNYV) genomic RNA (Deitzgen *et al*, 1989), known to contain the 4b gene of the virus, was amplified by PCR using the primers:

LNYV 1:5′-ATGGGATCCGTTATGCCAAGAAGAAGGA-3′; and

LNYV 2:5′-TGTGGATCCCTAACGGACCCGATG-3′

The first 9 nucleotide of these primers encode a BamHI site, the remaining nucleotides are homologous to sequences of the LNYV 4b gene.

Following amplification, the fragment was cloned into the EcoRI site of pCR2.1 (Stratagene). This EcoRI fragment was cloned into the EcoRI site of Cla pBC.PVY to create the intermediate plasmid pBC.PVY.LNYV which is shown in Figure 51.

Plasmid pBC.PVY.LNYV.PVY

The plasmid pBC.PVY.LNYV.YVP contains an interrupted direct repeat of PVY sequences. to create this plasmid a HpaI/HincII fragment from pSP72 was cloned into SmaI-digested pBC.PVY.LNYV and a plasmid containing the sense orientation isolated, a map of this construct is shown in Figure 52.

Plasmid pBC.PVY.LNYV.YVP$_\Delta$

The plasmid pBV.PVY.LNYV.YVP$_\Delta$ contains a partial interrupted palindrome of PVY sequences. One arm of the palindrome contains all the PVY sequences from pBC.PVY, the other arm contains part of the sequences from PVY, corresponding to sequences between the EcoRV and HincII sites of pSP72.PVY. To create this plasmid

an EcoRV/HincII fragment from pSP72.PVY was cloned into SmaI-digested pBC.PVY.LNYV and a plasmid containing the desired orientation isolated, a map of this construct is shown in Figure 53.

Plasmid pBC.PVY.LNYV.YVP

The plasmid pBC.PVY.LNYV.YVP contains an interrupted palindrome of PVY sequences. To create this plasmid a HpaI/HincII fragment from pSP72. was cloned into Sma-digested pBC.PVY.LNYV and a plasmid containing the antisense orientation isolated, a map of this construct is shown in Figure 54.

5. Control plasmids

Plasmids pART7.PVY & pART7.PVY

Plasmid pART7.PVY (Figure 55) was designed to express PVY sequences driven by the 35S promoter. This plasmid serves as a control construct in these experiments, against which all other constructs was compared. To generate this plasmid a ClaI/AccI fragment from ClapBC.PVY was cloned into ClaI-digested pART7 and a plasmid with expected to express a sense PVY sequence with respect to the PVY genome, was selected. Sequences consisting of the 35S promoter, PVY sequences and the OCS terminator were excised as a NotI fragment and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.

Plasmids pART7.35S.PVY.SCBV.O & pART27.35S.PVY.SCBV.O

Plasmid pART7.35S.PVY.SCBV.0 (Figure 56) was designed to act as a control for co-expression of multiple constructs from a single plasmid in transgenic plants. The 35S promoter was designed to express PVY sense sequences, whilst the SCBV promoter was empty. To generate this plasmid, the PVY fragment from Cla pBC.PVY was cloned as a XhoI/EcoRI fragment into XhoI/EcoRI-digested pART7.35S.SCBV.cass to create p35S.PVY.SCBV>O. Sequences consisting of the 35S promoter driving sense PVY sequences and the NOS terminator and the SCBV promoter and OCS terminator were excised as a NotI fragment and cloned into pART27, a plasmid with

the desired insert orientation was isolated and designated pART27.35S.PVY.SCBV.O.

Plasmids pART7.35S.O.SCBV.PVY & pART27.35S.O.SCBV.PVY

Plasmid pART27.35S.O.SCBV.PVY (Figure 57) was designed to act as an additional control for co-expression of multiple constructs from a single plasmid in transgenic plants. No expressible sequences were cloned behind the 35S promoter, whilst the SCBV promoter drove expression of a PVY sense fragment. To generate this plasmid, the PVY fragment from Cla pBC.PVY was cloned as a ClaI fragment into ClaI-digested pART7.35S.SCBV.cass, a plasmid containing PVY sequences in a sense orientation was isolated and designated p35S.O.SCBV.PVY. Sequences, consisting of the 35S promoter and NOS terminator, the SCBV promoter driving sense PVY sequences and the OCS terminator were excised as a NotI fragment and cloned into pART27, a plasmid with the desired insert orientation was isolated and designated pART27.35S.O.SCBV.PVY.

Plasmids pART7.35S.O.SCBV.YVP & pART7.35S.O.SCBV.YVP

Plasmid pART7.35S.O.SCBV.YVP (Figure 58) was designed to act as an additional control for co-expression of multiple constructs from a single plasmid in transgenic plants. No expressible sequences were cloned behind the 35S promoter, whilst the SCBV promoter drove expression of a PVY antisense fragment. To generate this plasmid, the PVY fragment from Cla pBC.PVY was cloned as a ClaI fragment into ClaI-digested p35S.SCBV.cass, a plasmid containing PCY sequences in an antisense orientation was isolated and designated p35S.O.SCBV.YVP. Sequences, consisting of the 35S promoter and NOS terminator, the SCBV promoter driving sense PVY sequences and the OCS terminator were excised as a NotI fragment and cloned into pART27, a plasmid with the desired insert orientation was isolated and designated pART27.35S.O.SCBV.YVP.

6. Test plasmids

Plasmids pART7.PVYx2 & pART27.PVYx2

Plasmid pART7.PVYx2 (Figure 59) was designed to express a direct repeat of PVY sequences driven by the 35S promoter in transgenic plants. To generate this plasmid, direct repeats from pBC.PVYx2 were cloned as a XhoI/BamHI fragment into XhoI/BamHI cut pART7. Sequences consisting of the 35 S promoter, direct repeats of PVY and the OCS terminator were excised as a NotI fragment from pART7.PVYx2 and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVYx2.

Plasmids pART7.PVYx3 & pART27.PVYx3

Plasmid pART7.PVYx3 (Figure 60) was designed to express a direct repeat of three PVY sequences driven by the 35S promoter in transgenic plants. To generate this plasmid, direct repeats from pBC.PVYx3 were cloned as a XhoI/BamHI fragment into XhoI/BamHI cut pART7. Sequences consisting of the 35S promoter, direct repeats of PVY and OCS terminator were excised as a NotI fragment from pART.PVYx3 and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVYx3.

Plasmids pART7.PVYx4 & pART27.PVYx4

Plasmid pART7.PVYx4 (Figure 61) was designed to express a direct repeat of four PVY sequences driven by the 35S promoter in transgenic plants. To generate this plasmid, direct repeats from pBC.PVYx4 were cloned as a XhoI/BamHI fragment into xhoI/BamHI cut pART7. Sequences consisting of the 35S promoter, direct repeats of PVY and the OCS terminator were excised as a NotI fragment from pART7.PVYx3 and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVYx3.

Plasmids pART7.PVY.LNYV.PVY & pART27.PVY.LNYV.PVY

Plasmid pART7.PVY.LNYV.PVY (Figure 62) was designed to express the interrupted direct repeat of PVY sequences driven by the 35S promoter in transgenic plants. This construct was prepared by cloning the interrupted direct repeat of PVY from

pBC.PVY.LNYV.PVY as a XhoI/XbaI fragment into pART7 digested with XhoI/XbaI. Sequences consisting of the 35S promoter, the interrupted direct repeat of PVY sequences and the OCS terminator were excised from pART7.PVY.LNYV.PVY as a NotI fragment and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVY.LNYV.PVY.

Plasmids pART7.PVY.LNYV.YVP$_\Delta$ & pART27.PVY.LNYV.YVP$_\Delta$

Plasmid pART7.PVY.LNYV.YVP$_\Delta$ (Figure 63) was designed to express the partial interrupted palindrome of PVY sequences driven by the 35S promoter in transgenic plants. This construct was prepared by cloning the partial interrupted palindrome of PVY sequences from pBC.PVY.LNYV.YVP$_\Delta$ as a XhoI/XbaI fragment into pART7 digested with XhoI/XbaI. Sequences consisting of the 35S promoter, the partial interrupted palindrome of PVY sequences and the OCS terminator were excised from pART7.PVY.LNYV.YVP$_\Delta$ as a NotI fragment and cloned into NotI-digested pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVY.LNYV.YVP.

Plasmids pART7.PVY.LNYV.YVP & pART27.PVY.LNYV.YVP

Plasmid pART7.PVY.LNYV.YVP (Figure 64) was designed to express the interrupted palindrome of PVY sequences driven by the 35S promoter in transgenic plants. This construct was prepared by cloning the interrupted palindrome of PVY sequences from pBC.PVY.LNYV.YVP$_\Delta$ as a XhoI/XbaI fragment into pART7 digested with XhoI/XbaI. Sequences consisting of the 35S promoter, the interrupted palindrome of PVY sequences and the OCS terminator were excised from pART7.PVY.LNYV.YVP as a NotI fragment and cloned into pART27, a plasmid with the desired insert orientation was selected and designated pART27.PVY.LNYV.YVP.

Plasmids pART7.35S.PVY.SCBV.YVP & pART27.35S.PVY.SCBV.YVP

Plasmid pART7.35S.PVY.SCBV.YVP (Figure 65) was designed to co-express sense and antisense constructs in transgenic plants. To generate this plasmid the PVY

fragment from Cla pBC.PVY was cloned as a XhoI/EcoRI fragment into xhoI/EcoRI-digested p35S.SCBV.O.SCBV.YVP. Sequences, consisting of the 35S promoter driving sense PVY sequences and the NOS terminator and the SCBV promoter driving antisense PVY and the OCS terminator were excised as a NotI fragment and cloned into pART27, a plasmid with the desired insert orientation was isolated and designated pART27.35S.PVY.SCBV.YVP.

Plasmids pART7.35S.PVYx3.SCBV.YVPx3 & pART27.35S.PVYx3.SCBV.YVPx3

Plasmid pART7.35S.PVYx3.SCBV.YVPx3 (Figure 66) was designed to co-express sense and antisense repeats of PVY in transgenic plants. to generate this plasmid, the intermediate pART7.35S.O.SCBV.YVPx3 was constructed by cloning the triple direct PVY repeat from ClapBC.PVYx3 as a ClaI/AccI fragment into Cla-digested p35S.SCBV.cass and isolating a plasmid with an antisense orientation. for p35S.PVYx3.SCBV.YVPx3 the triple direct PVY repeat from Cla pBC.PVYx3 was cloned as a KpnI/SmaI fragment into KpnI/SmaI-digested p35S.O.SCBV.YVPx3 to create p35S.PVYx3.SCBV.YVPx3. Sequences including both promoters, terminators and direct PVY repeats were isolated as a NotI fragment and cloned into pART27. A plasmid with an appropriate orientation was chosen and designated pART27.35S.PVYx3.SCBV.

Plasmids pART7.PVYx3.LNYV.YVPx3 & pART27.PVYx3.LNYV.YVPx3

Plasmid pART7.PVYx3.LNYV.YVPx3 (Figure 67) was designed to express triple repeats of PVY sequences as an interrupted palindrome. To generate this plasmid an intermediate, pART7x3.PVY.LNYV.YV was constructed by cloning a PVY.LNYV.YVP fragment from pBC.PVY.LNYV.YVP as an AccI/ClaI fragment into the plasmid pART7.PVYx2. pART7.35S.PVYx3.LNYV.YVPx3, was made by cloning an additional PVY direct repeat from pBC.PVYx2 as an AccI/ClaI fragment into ClaI digested pART7x3.PVY.LNYV.YVP. Sequences from pART7.35S.PVYx3.LNYV.YVPx3, including the 35S promoter, all PVY sequences and the OCS terminator were excised as a NotI fragment and cloned into NotI-digested pART27, a plasmid with an

appropriate orientation was chosen and designated pART27.35S.PVYx3.LNYV.

Plasmids pART7.PVY multi & pART27.PVY multi

Plasmid pART7.35S.PVY multi (Figure 68) was designed to express higher order direct repeats of regions of PVY sequences in transgenic plants. Higher order direct repeats of a 72 bp of the PVY Nia region from PVY were prepared by annealing two partially complementary oligonucleotides as follows:

PVY1:

5′-TAATGAGGATGATGTCCCTACCTTTAATTGGCAGAAATTTCTGTGGAAAGACAG GGAAATCTTTCGGCATTT-3′; and

PVY2:

5′-TTCTGCCAATTAAAGGTAGGGACATCATCCTCATTAAAATGCCGAAAGATT TCCCTGTCTTTCCACAGAAAT-3′

The oligonucleotides were phosphorylated with T4 polynucleotide kinase, heated and cooled slowly to permit self-annealing, ligated with T4 DNA ligase, end-filled with Klenow polymerase and cloned into pCR2.1 (Invitrogen). Plasmids containing multiple repeats were isolated and sequences were cloned as EcoRI fragments in a sense orientation into EcoRI-digested pART7, to create the intermediate pART7.PVY multi. to create pART27.PVY multi, the 35S promoter, PVY sequences and the OCS terminator were excised as a NotI fragment and cloned into NotI-digested pART27. A plasmid with an appropriate insert orientation was isolated and designated pART27.PVY multi.

EXAMPLE 6

Inactivation of virus gene expression in mammals

Viral immune lines are created by expressing viral sequences in stably transformed cell lines.

In particular, lytic viruses are used for this approach since cell lysis provides very simple screens and also offer the ability to directly select for potentially rare transformation events which might create viral immunity. Sub-genomic fragments derived from a simple single stranded RNA virus (Bovine enterovirus - BEV) or a complex double stranded DNA virus, Herpes Simplex Virus I (HSV I) are cloned into a suitable vector and expressed in transformed cells. Mammalian cell lines are transformed with genetic constructs designed to express viral sequences driven by the strong cytomegalovirus (CMV-IE) promoter. Sequences utilised include specific viral replicase genes. Random "shotgun" libraries comprising representative viral gene sequences, may also be used and the introduced dispersed nucleic acid molecule, to target the expression of virus sequences.

Exemplary genetic constructs for use in this procedure, comprising nucleotide sequences derived from the BEV RNA-dependent RNA polymerase gene, are presented herein.

For viral polymerase constructs, large numbers (approximately 100) of transformed cell lines are generated and infected with the respective virus. For cells transformed with shotgun libraries very large numbers (hundreds) of transformed lines are generated and screened in bulk for viral immunity. Following virus challenge, resistant cell lines are selected and analysed further to determine the sequences conferring immunity thereon.

Resistant cell lines are supportive of the ability of the introduced nucleotide sequences to inactivate viral gene expression in a mammalian system.

Additionally, resistant lines obtained from such experiments are used to more precisely define molecular and biochemical characteristics of the modulation which is observed.

EXAMPLE 8

Induction of virus resistance in transgenic plants

Agrobacterium tumefaciens, strain LBA4404, was transformed independently with the constructs pART27.PVY, pART27.PVYx2, pART27.PVYx3, pART27.PVYx4, pART27.PVY.LNYV.PVY, pART27.PVY.LNYV.YVP$_{\Delta}$, pART27.PVY.LNYV.YVP, pART27.35S.PVY.SCBV.O, pART27.35S.O.SCBV.PVY, pART27.35S.O.SCBV.YVP, pART27.35S.PVY.SCBV.YVP, pART27.35S.PVYx3.SCBV.YPVx3, pART27.PVYx3.LNYV.YVPx3 and pART27.PVYx10, using tri-parental matings. DNA mini-preps from these strains were prepared and examined by restriction with NotI to ensure they contained the appropriate binary vectors.

Nicotiana tabaccum (cultivar W38) were transformed with these *Agrobacterium* strains using standard procedures. Putative transformed shoots were excised and rooted on media containing kanamycin. Under these conditions we have consistently observed that only transgenic shoots will root on kanamycin plates. Rooted shoots were transferred to soil and allowed to establish. After two to three weeks, vigorous plants with at least three sets of leaves were chosen and infected with PVY.

Viral inoculum was prepared from W38 tobacco previously infected with the virus, approximately 2 g of leaf material, showing obvious viral symptoms were ground with carbarundum in 10 ml of 100mM Na phosphate buffer (pH 7.5). the inoculum was diluted to 200 ml with additional Na phosphate buffer. Two leaves from each transgenic plant were sprinkled with carbarundum, then 0.4 ml of inoculum was applied to each leaf and leaves rubbed fairly vigorously with fingers. Using this procedure 100% of non-transgenic control plants were infected with PVY.

To assay for viral resistance and immunity transgenic plants are monitored for symptom development. The PVY strain (PVY-D, an Australian PVY isolate) gives obvious symptoms on W38 tobacco, a vein clearing symptom is readily observed on the two leaves above the inoculated leaves, subsequent leaves show uniform chlorotic lesions. Symptom development was monitored over a six week period.

Transgenic lines were described as resistant if they showed reduced viral symptoms, which manifests as a reduction in the leaf are showing chlorotic lesions. Resistance ranges from very strong resistance where only a few viral lesions are observed on a plant to weak resistance which manifects as reduced symptoms on leaves that develop late in plant growth.

Transgenic plants which showed absolutely no evidence of viral symptoms were classified as immune. To ensure these plants were immune they were re-inoculated with virus, most plants remained immune, the few that showed symptoms were re-classified as resistant.

For plant lines generated Southern blots are performed, resistance in subsequent generations is monitored to determine that resistance/immunity is transmissable. Additionally, the breadth of viral resistance is monitored by challenging lines with other PVY strains, to determine whether host range susceptibility is modified.

Results from these experiments are described in Table 2 . These data indicate that constructs comprising tandem repeats of target gene sequence, either in the configuration of palindromes, interrupted palindromes as direct repeat sequences, are capable of conferring viral resistance and/or immunity in transgenic plants.

Accordingly, such inverted and/or direct repeat sequences modulate expression of the virus target gene in the transgenic plant.

Constructs combining the use of direct and inverted repeat sequences, namely pART27.35S.PVYx3.SCBV.YVPx3 and pART27.PVYx3.LNYV.YVPx3, are also useful in modulating gene expression.

EXAMPLE 9

Inactivation of Galt in animal cells

To assay for Galt inactivation, porcine PK2 cells were transformed with the relevant constructs. PK2 cells constitutively express Galt enzyme, the activity of which results in the addition of a variety of α-1,3-galactosyl groups to a range of proteins expressed on the cell surface of these cells. Cells were transformed using lipofectin and stably transformed lines were selected using genetecin.

As an initial assay cell lines were probed for the presence of the Galt-encoded epitope, i.e. α-1,3-galactosyl moieties decorating cell surface proteins, using the lectin IB4. IB4 binding was assayed either *in situ* or by FACS sorting.

For *in situ* binding, cells were fixed to solid supports with cold methanol for 5 mins, cells were rinsed in PBS (phosphate buffered saline) and non-specific IB4 binding was blocked with 1% BSA in PBS for 10 mins. Fixed cells were probed using 20 ug/ml IB4-biotin (Sigma) in 1% BSA, PBS for 30 mins at room temperature, cells were washed in PBS then probed with a 1:200 dilution of ExtrAvidin-FITC (Sigma) in PBS for 30 mins followed by further rinses in PBS. Cells were then examined using fluorescence microscopy, under these conditions the outer surface of PK2 control cells uniformly stained green.

For FACS analysis, cells were suspended after treatment with trypsin, washed in HBSS/Hepes (Hank's buffered saline solution with 20 mM Hepes, pH7.4) and probed with 10 ug/ml IB4-biotin (Sigma) in HBSS/Hepes for 45 mins at 4°C. Cells were washed in HBSS/Hepes, probed with a 1:200 dilution of ExtrAvidin-FITC (Sigma) in HBSS/Hepes for 45 mins at 4°C at and rinsed in cold HBSS/Hepes prior to FACS

sorting.

Using this approach transformed cell lines are assayed for Galt inactivation and quantitative assessment of construct effectiveness is determined. Moreover cell lines showing Galt inactivation are isolated and subject to further molecular analyses to determine the mechanism of gene inactivation.

PLASMID CONSTRUCT	No. OF PLANTS TESTED	PERCENTAGE OF PLANTS SHOWING SPECIFIED PHENOTYPE		
		SUSCEPTIBLE	IMMUNE	RESISTANT
pART27.PVY	19	16	1	2
pART27.PVYx2	13	5	4	4
pART27.PVYx3	21	2	5	14
pART27.PVYx4	21	5	7	9
pART27.35S.PVY.SCBC.0	25	8	0	17
pART27.35S.O.SCBV.PVY	22	8	0	14
pART27.35S.O.SCBV.YVP	18	14	0	4
pART27.35S.PVY.SCBV.YVP	17	3	8	6
pART27.PVY.LNYV.PVY	26	18	2	6
pART27.PVY.LNYV.YVP	20	6	10	4
pART27.PVY.LNYV.YVP$_{\Delta}$	18	7	11	0

REFERENCES

1. An *et al.* (1985) *EMBO J 4:*277-284.
2. Armstrong, *et al.Plant Cell Reports 9:* 335-339, 1990.
3. Ausubel, F.M. *et al.*(1987) *In:* Current Protocols in Molecular Biology, Wiley Interscience (ISBN 047140338)..
4. Chalfie,M. *et al* (1994) *Science* **263**: 802-805.
5. Christensen, A.H. and Quail, P.H. (1996) *Transgenic Research* **5**: 213-218.
6. Christou, P., *et al. Plant Physiol 87:* 671-674, 1988.
7. Cormack, B. *et al* (1996) *Gene* **173**: 33-38.
8. Crossway *et al., Mol. Gen. Genet. 202:*179-185, 1986.
9. Dorer, D.R., and Henikoff, S. (1994) *Cell* **7**: 993-1002.
10. Fromm *et al. Proc. Natl. Acad. Sci. (USA) 82:*5824-5828, 1985.
11. Gleave, A.P. (1992) *Plant Molecular Biology* **20**:1203-1207.
12. Hanahan, D. (1983) *J. Mol.Biol.* **166**: 557-560.
13. Herrera-Estella *et al., Nature 303:* 209-213, 1983a.
14. Herrera-Estella *et al.,EMBO J. 2:* 987-995, 1983b.
15. Herrera-Estella *et al. In:* Plant Genetic Engineering, Cambridge University Press, N.Y., pp 63-93, 1985.
16. Inouye, S. and Tsuji, F.I. (1994) *FEBS Letts.* **341**: 277-280.
17. Jackson, I.J. (1995) *Ann. Rev. Genet.* **28**: 189-217.
18. Krens, F.A., *et al., Nature 296:* 72-74, 1982.
19. Kwon, B.S. *et al.* (1988) *Biochem. Biophys. Res. Comm.* **153**:1301- 1309.
20. Pal-Bhadra, M. *et al.* (1997) *Cell* **90**: 479-490.
21. Paszkowski *et al., EMBO J. 3:*2717-2722, 1984
22. Prasher, D.C. *et al.* (1992) *Gene* **111**: 229-233.
23. Sanford, J.C., *et al., Particulate Science and Technology 5:* 27-37, 1987.